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GROWTH FACTORS IN ALCOHOLIC EXTRACTS OF CHICK EMBRYOS[1]

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INTRODUCTION

The lack of a defined medium for animal cells *in vitro* constitutes at present a strategic roadblock to the analysis of growth processes at the cellular level as well as to the effective use of cell populations for the study of adaptation, carcinogenesis, and biological problems in general. In a preceding paper (Harris, 1952) the historical aspects of this problem have been outlined briefly and current approaches noted. No combination of known crystalloidal growth factors is as yet adequate to promote protein synthesis (Gerarde, Jones, and Winnick, 1951) or increase in cell number (Evans, Shannon, Waltz, Bryant, Sanford, and Earle, 1952) in tissue cultures. The inadequacy of synthetic nutrients currently available may reflect a protein requirement for animal tissue cells *in vitro* and/or additional specific growth factors. The missing components, whatever their nature, are found in tissue extracts and a variety of naturally occurring materials. The pioneer studies of Carrel showed that certain cells types will proliferate indefinitely in a plasma medium containing embryo extract, and that extracts of adult tissues as well as leucocytes also possess growth-promoting activity (Parker, 1950). Recently Gerarde and associates (1952) have demonstrated extensive net protein synthesis for chick tissues growing in roller tubes in the presence of 25% chick embryo extract. Other experiments indicate that mouse fibroblasts increase in number when maintained as cell suspensions in chick embryo extract and saline alone, although greater increases are obtained if horse serum is present in the medium as well (Evans *et al.*, 1952). These observations suggest that direct

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fractionation of embryo extract and serum may lead to a more definite characterization of the growth factors required for fibroblasts and other cells *in vitro*.

Dialyzable fractions from embryo extract have been studied by Fischer and co-workers (Fischer, 1946; Fischer, Astrup, Ehrensvard, and Oehlenschlager, 1948), and recently by Earle and associates (Sanford, Waltz, Shannon, Earle, and Evans, 1952). Fischer dialyzed saline extracts of calf embryo muscle and obtained an active material by evaporating the dialysate. Although this preparation was fractionated with barium salts and other procedures the nature of the active factors was not ascertained. Earle and co-workers prepared ultrafiltrates of chick embryo extract and horse serum, using a Simms apparatus and cultured suspensions of mouse fibroblasts in various combinations of the ultrafiltrates and residues. Nuclear counts served as a growth index. The results indicated that in the presence of horse serum the ultrafiltrate of chick embryo extract is more effective than embryo extract residue and almost as potent as whole unfiltered chick embryo extract for growth of the cell type used. The nature of the active substances in the ultrafiltrates was not investigated.

The present paper deals also with dialyzable growth factors in chick embryo extract. Fractions containing these agents have been obtained by direct alcoholic extraction of fresh embryos. Preliminary separations and dialysis were carried out in 70% ethanol to avoid losses or alteration of substances originally present as a result of enzymatic or bacterial action. From the nature of the results obtained it seems probable that the active factors concerned are similar to those studied in the preceding experiments of Fischer and in the recent investigations of Earle's group.

For assay of preparations obtained by alcoholic fractionation, we have employed dialyzed media according to the method first devised by Fischer (1941) and subsequently modified by the present author (Harris, 1952). In this procedure the classic plasma coagulum is used with a supernatant containing serum and embryo extract. Each component is subjected to extensive dialysis against a buffered saline before combination in the final medium. In the resulting cultures outgrowth proceeds but at a greatly reduced level with characteristic alterations in cell morphology and colonial pattern (Harris, 1952). This pattern of changes is readily reversed by the addition of dialysate from chick embryo extract. Dialyzed media thus afford a sensitive

test for the active factors concerned in the dialysate fraction and have been used throughout in the work to be described.

Material and Methods

Preparation of media, dialysis procedures, and culturing operations have been described in detail in a previous paper (Harris, 1952) to which reference may be made for a complete account of methods used. Chicken plasma and chick embryo extract were prepared directly and fresh horse serum obtained commercially from Cutter Laboratories, Berkeley, California. The media were then dialyzed in 25 ml. aliquots for 8 days. Each aliquot in a dialysis sac was suspended in a 2 liter Erlemeyer Flask containing sterile F-2 dialyzing solution. The composition of this fluid is as follows:

F-2 solution:

NaCl	7.33 grams
KCl	0.38 grams
$CaCl_2$ (anhydrous)	0.13 grams
$MgCl_2 \cdot 6H_2O$	0.21 grams
$NaHCO_3$	1.40 grams
KH_2PO_4	0.11 grams
Na_2HPO_4 (anhydrous)	0.30 grams
Distilled water	1,000 ml.

The dialyzing fluid was changed daily (except Sunday) and the final media tested for sterility before use. Explants were derived from fresh 12-day chick heart and were maintained in Carrel flasks at 38° C. In starting the cultures, 0.5 ml. dialyzed chicken plasma was placed in each flask and subsequently diluted to 1.5 ml. by the addition of a clotting fluid which was also used to adjust the composition of the coagulum to match that of the supernatant. After clotting, the medium was completed by adding 1.0 ml. supernatant fluid and the flasks sealed with rubber stoppers. In some experiments a mixture of 40% dialyzed horse serum and 60% Gey's solution served as a supernatant. In this case the concentration of dialyzed saline embryo extract in the clotting fluid was reduced to a minimum level required for satisfactory clotting (0.02 ml. EE_{50} per flask). This medium containing no further EE_{50} in the supernatant fluid will be referred to as dilute serum. In other experiments an embryo extract-serum-

saline mixture was used as supernatant, consisting specifically of 15% dialyzed saline embryo extract (EE_{50}), 40% dialyzed horse serum, and 45% Gey's solution.

In all cases supplements to be tested were incorporated in the saline fraction, as were the following at levels appropriate to give the final concentration noted: 0.1% D-glucose, 0.1% $NaHCo_3$ 100 units/ml. sodium penicillin G (Merck), 50 units/ml. dihydrostreptomycin (Pfizer), and 0.002% phenol red. The supernatant fluids were drawn off and replaced twice weekly without washing the cultures. Routine areal tracings made at 15X with a projectoscope. All areal measurements were expressed in terms of final minus initial area and the data treated statistically.

Experimental Work

Effect of alcoholic fractions from chick embryos

Preparation of extracts.—Embryos incubated for 12 days were excised from their membranes and after removal of legs and eyes were washed in distilled water, blotted briefly, and weighed in homogenizer tubes. Subsequently 2.15 ml. absolute ethanol per gram wet weight were added and the embryos ground thoroughly with a motor-driven pestle. The resulting homogenates were stored at 4° C. for 24 hours and then centrifuged to yield a clear yellow supernatant containing approximately 70% ethanol. Most of the lipids and alcohol-soluble proteins were subsequently removed by extracting the supernatant four times successively with equal volumes of chloroform in a separatory funnel followed by centrifugation. The final supernatant was evaporated to dryness over $CaCl_2$ with very gentle heat from an infra-red lamp. The residue was reconstituted by adding 70% ethanol (0.3 ml. per gram initial wet weight), and after centrifugation 20 ml. aliquots were placed in dialysis sacs. Dialysis was carried out against equal volumes of 70% ethanol in a cold room using a Boerner shaker. Four changes of dialyzing fluid were made over a period of three days. The non-dialyzable fraction (within the sacs) was then usually discarded and the dialysates combined for evaporation to dryness over $CaCl_2$ with very gentle heat from an infra-red lamp. For purposes of initial testing, however, a few aliquots of the non-dialyzable fraction were dialyzed for three additional days on the Boerner shaker against a number of 2 liter changes

of 70% ethanol. The contents of the sacs (non-dialyzable) were then pooled for evaporation and assay along with the dialysates previously prepared.

The evaporated residues from the dialyzable fraction represented 9-10 mg. per gram initial wet weight of embryos. Approximately 0.8 mg. per gram wet weight remained in the non-dialyzable fraction. For assay purposes these materials were reconstituted with glucose-free Gey's solution, sterilized by passage through a sintered glass filter (ultrafine) and used at final concentrations as shown in Table 1.

Activity of alcoholic fractions.—When dialyzed media were used for assay, the addition of dialysate from the original alcoholic extract produced conspicuous and clear-cut changes in appearance of cells, pattern of outgrowth and surface area. These changes could be obtained with media containing high levels of saline embryo extract (embryo extract-serum-saline) as well as with minimal levels of saline embryo extract (dilute serum). Table 1 summarizes a representative array of data obtained.

In unsupplemented serum cultures only a narrow ring of cells developed at the margin of the explant. As previously described (Harris, 1952) these cells formed an open meshwork and after a few days were refractile, vacuolated and jagged in outline. The cell picture in similar cultures supplemented with dialysate from ethanol extracts provided a marked contrast. In this case a dense outgrowth of broad flat cells appeared, non-refractile and without conspicuous inclusions. A prominent visible increase in number of cells in the marginal region was reflected in the gross rise in surface area shown in Table 1. In general the use of areal measurements with serum cultures is not practicable owing to the high variability of individual outgrowths (Parker, 1950). In the experiments at hand, however, the differential produced by supplementation was gross in comparison with individual variation and thus provided a convenient index of stimulation. A second index was provided in some cases by analyses for lactic acid in the terminal supernatant fluids removed from the flasks after the 10-day culture period. These were pooled for each series of six cultures and aliquots used for determinations by the method of Barker and Summerson (1941) using the Beckman spectrophotometer. The data shown in Table 1 indicate that stimulation of outgrowth produced by added dialysate is accompanied by an increased level of glycolysis.

TABLE 1

OUTGROWTH OF CHICK HEART FIBROBLASTS IN DIALYZED MEDIA SUPPLEMENTED WITH ALCOHOLIC EXTRACTS FROM 12-DAY EMBRYOS

Designation of medium*	Composition of supernatant	Fractions of 70% ethanol extract added: Dialysate Mg./ml.	Fractions of 70% ethanol extract added: Non-dialysable fraction, Mg./ml.	Areal increase† per explant at 10 days, mm^2	Significance of areal differences from outgrowth in unsupplemented medium	Lactic acid‡ in supernatants at 10 days, α/ml.
Dilute serum		—	—	13.5± 0.75	————	227
	40% dialyzed horse serum	2.2	—	63.3±11.3	P = <0.01	471
		—	0.2	21.7± 6.2	P = <0.2	—
	60% Gey's solution	2.2	0.2	57.1± 9.8	P = <0.01	—
		Undialyzed alcoholic extract, 2.5 Mg./ml.		55.3± 3.8	P = <0.01	—
Embryo-extract-serum-saline	15% dialyzed saline embryo extract	—	—	64.1± 6.1	————	538
		2.2	—	138.4± 8.4	P = <0.01	736
	40% dialyzed horse serum	—	0.2	53.5± 4.8	P = <0.2	—
		2.2	0.2	126.6± 5.1	P = <0.01	—
	45% Gey's solution	Undialyzed alcoholic extract, 2.5 Mg./ml.		102.9± 6.1	P = <0.01	—

*All explants maintained in dialyzed plasma clot in Carrel flasks.

†Mean value and standard error (Snedecor, 1946). Six cultures in each series.

‡Average value for duplicate determinations.

In other experiments serum cultures were supplemented with the non-dialyzable fraction of the original ethanol extract. These cultures were indistinguishable in cell morphology and colonial pattern from cultures made with unsupplemented medium and showed only minor differences in surface area. The combination of dialyzable and non-dialyzable fractions from the ethanol extract did not augment the effects produced by the dialysate alone.

Parallel results to those just described were obtained when an embryo-extract-serum medium was used instead of dilute serum. As previously described (Harris, 1952) the basal outgrowths in unsupplemented medium were larger than in serum cultures but were clearly still suboptimal in micoscopic appearance as well as areal spread. The cells showed a loose "brush-heap" arrangement in the outgrowth zone and became conspicuously refractile and vacuolated in a few days. The addition of dialysate produced massive cell populations in the outgrowth zone with increased surface area and glycolytic activity. These outgrowths remained relatively clear, free from the conspicuous inclusions characteristic of the unsupplemented media, and in general closely resembled outgrowths in undialyzed media of similar composition. The average level of outgrowth in embryo-extract-serum cultures fortified with dialysate was higher than in the comparable supplemented serum cultures and the thickness of the outgrowth zone was noticeably greater. Glycolysis was also higher than in comparable serum cultures although the differential produced by added dialysate was less. These differences may reflect the presence of the protein components of embryo extract in the embryo extract-serum-saline medium and their essential absence in the dilute serum medium.

The non-dialyzable portion of the ethanol extracts was without stimulating effect in the embryo-extract-serum medium when tested alone as well as in combination with the dialysate fraction. No sign of activity from the non-dialyzable fraction was observed microscopically or in terms of surface area (Table 1). Although this supplement has not been defined and contains unknown materials in addition to pigments and small amounts of protein, it is apparent that these components are inert or slightly inhibiting with activity residing entirely in the dialyzable fraction. Accordingly further investigations of the alcoholic extract as outlined below will deal exclusively with crude dialysate preparations.

Characteristics of the crude dialysate.—The absence of protein in concentrated solutions of dialysate was confirmed by negative tests with hot and cold 20% trichloracetic acid as well as with tungstic acid. Biuret tests were also negative. Molisch and orcinol tests were strongly positive indicating the presence of carbohydrate, especially pentose. The occurrence of nucleic acid derivatives or related compounds was apparent from ultraviolet absorption spectra made with saline solutions of dialysate (pH 7.6). Readings with the Beckman spectrophotometer of optical densities were made at intervals of 5 mμ over the range 220-320 mμ. A prominent peak was found at approximately 260 mμ with a minimum near 235 mμ, rising sharply again below 230 mμ. The presence of large amounts of free amino acids in the dialysate was confirmed with ninhydrin.

Experiments were performed to determine the solubility of active factors from the dialysate in organic solvents. Acetone, chloroform, absolute ethanol, and ether-alcohol (1 part absolute diethyl ether : 3 parts absolute ethanol) were studied in separate series. Aliquots of approximately 100 mg. dry dialysate were extracted repeatedly in small flasks with 25 ml. solvent. Extracts and residues were then evaporated to dryness over $CaCl^2$, reconstituted with Gey's solution and assayed in culture at levels comparable to those described in the preceding section. In each case the residue was as active as the original dialysate while the solvent fractions were inert but not inhibiting. Further assays were conducted to determine the solubility of dialysate in ethanol solutions of ascending concentration. These results indicated complete solubility of active factors up to levels of 70-75% ethanol, the solubility declining above this point without a sharp break.

Additional experiments were designed to investigate the stability of dialysate factors to heat and acid. Neutral solutions of dialysate were heated for 10 minutes at 100° C. in a water bath without diminution in activity. Comparable preparations autoclaved at 15 lbs. for 15 minutes showed prominent though not complete loss of activity. When autoclaved dialysate was added to assay cultures the characteristic stimulation of outgrowth and glycolysis was markedly reduced and the cells were refractile and vacuolated. A sharp reduction in activity was also obtained by dissolving the dry dialysate in 1N-HCl and heating to 100° C. in a water bath for 10 minutes. For assay purposes this preparation was neutralized with NaOH, diluted

to an isotonic level, sterilized by filtration and finally added to give the equivalent of 2.5 mg. extract per ml. in the completed medium. The appearance of these cultures was similar to those supplemented with autoclaved dialysate. Another dialysate preparation was dissolved in 8N-HCl and autoclaved for 3½ hours at 15 lbs. The resulting solution was evaporated over NaOH and re-evaporated twice after reconstitution with distilled water. Final reconstitution was made with Gey's solution, the pH level adjusted to 7.6 and humin removed during sterilization by filtration. This preparation was nearly inert but not inhibiting unless added in high concentration. In the serum cultures used for assay a small increase in area and cell number was apparent in comparison to unsupplemented controls but the appearance of the cells was essentially the same in both series.

The data outlined above thus suggest that the active dialysate factors are not protein or lipid in nature, are partially heat-stable, but are largely destroyed by acid hydrolysis.

Supplementation of Dialyzed Media With Known Growth Factors

Amino acids.—The question arises whether the activity of crude dialysate can be accounted for on the basis of free amino acids known to be present. This possibility has been affirmed by Fischer in a series of earlier investigations with dialyzed media (see summaries in Fischer, 1946; Fischer *et al.*, 1948). To test the ability of added amino acids to duplicate the effects of crude dialysate in the present experiments, a mixture of 20 pure amino acids was prepared using proportions similar to those adopted by Morgan, Morton, and Parker (1950) for the culture of chick fibroblasts in synthetic media. The naturally occurring (L) forms were used except in the case of methionine, serine, threonine, valine, isoleucine, and alanine for which DL forms only were available. While this point could conceivably be significant, it may be emphasized that Morgan, Morton, and Parker found no differences in using a similar mixture and one made up entirely of L forms. For assay purposes a dialyzed embryo extract-serum-saline medium was employed as described above, with additions of the amino acid mixture ranging from 50 γ/ml. to 2,000 γ/ml.

The cell picture in assay cultures was either similar to that of unsupplemented control cultures (50-100 γ/ml.) or showed definite signs of inhibition (250-2,000 γ/ml.). With high levels of added

amino acids the cells were scattered, excessively vacuolated, and abnormal in appearance. Figure 1 gives a comparison of areal increases obtained. The only sign of activity from supplementary amino acids was a possible slight areal increase at levels of 50 γ/ml. although the difference noted is well within the bounds of sampling error. At concentrations of 250 γ/ml. or more, areas were significantly reduced in comparison to unsupplemented controls.

Failure of amino acid supplements to stimulate outgrowth in dialyzed media suggests that although the concentration of these agents may be reduced by initial dialysis they are replaced in part by the activity of proteolytic enzymes in the cells or medium. To test

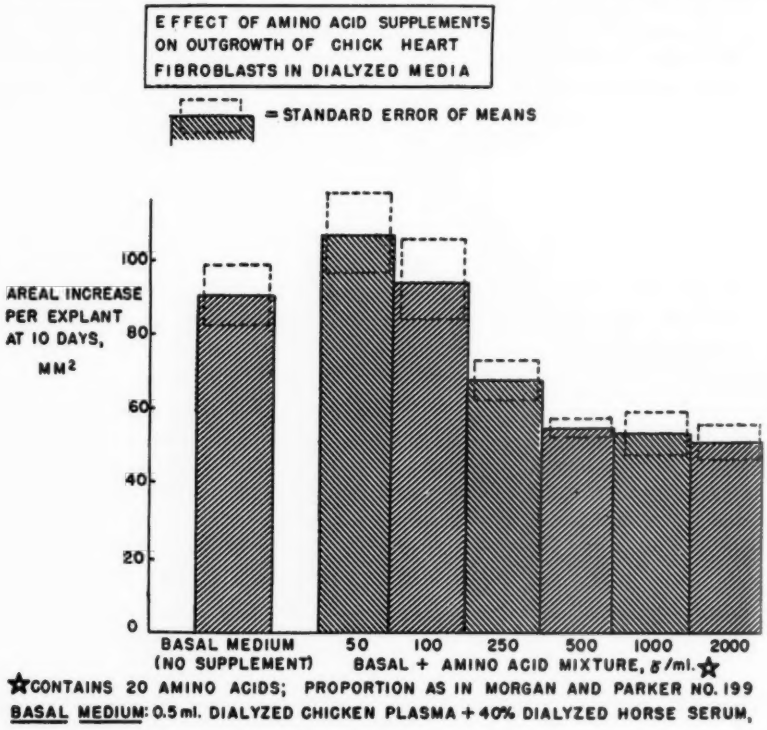

FIGURE 1

this possibility a series of measurements were made of free amino nitrogen in deproteinized supernatants, from flasks containing cultures as well as from flasks incubated with a blank plasma clot and associated fluid phase. Determinations were carried out according to the method of Frame, Russell, and Wilhelmi (1943) as modified by Russell (1944), using the Beckman spectrophotometer. An embryo extract-serum-saline medium was employed and phenol red omitted to avoid interference in subsequent measurements. Twelve cultures and twelve blank flasks were incubated over the usual 10-day period. At the beginning of the experiment and at 3, 7, and 10 days the supernatants were pooled from pairs of similar flasks and aliquots set

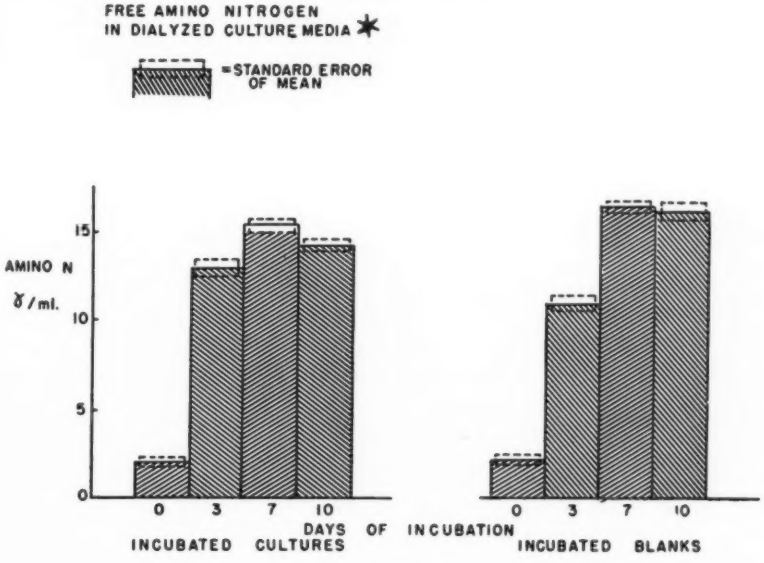

FIGURE 2

aside for determination. Figure 2 indicates the result of this experiment. Both in incubated blanks and in cultures there was well-defined rise in free amino nitrogen from the low level initially present in the stock dialyzed medium. These data support the assumption that

degradation of proteins or other substrates tends to restore in part the free amino acids found in media components before dialysis.

Nucleic acid derivatives.—Since the crude dialysate shows a prominent absorption peak at 260 mμ in the ultraviolet it seemed possible that active factors might be found among the various nucleic acid derivatives. Previous studies (Kutsky and Harris, 1952) had shown that nuclear fractions isolated centrifugally from chick embryos were

TABLE 2

EFFECT OF RIBONUCLEIC ACID DERIVATIVES ON OUTGROWTH OF CHICK HEART FIBROBLASTS IN DIALYZED MEDIA

Medium*	Supplement	Concentration of supplement in medium, γ/ml.	Areal increase† per explant at 10 days, mm²
0.5% EE_{50}	None (control)	—	17.1±4.6
	Ribonucleic acid: (Schwartz)	100	17.9±4.5
	Mononucleotides: (adenylic, cytidylic, guanylic, uridylic acids)	1,000	9.5±2.7
		100	11.0±1.1
		10	16.3±2.6
	Nucleosides: (adenosine, cytidine, guanosine, uridine)	1,000	7.9±1.2
		100	18.1±4.2
		10	15.8±1.9
15% EE_{50}	None (control)	—	67.7±1.9
	Free bases: (adenine, guanine. cytosine, uracil. + thymine, xanthine, hypoxanthine)	100	31.0±1.8
		10	55.6±3.2

**Total medium:* 0.5 ml. dialyzed chicken plasma, 15% or 0.5% dialyzed EE_{50}, 45% or 59.5% Gey's solution, 40% dialyzed horse serum.

†Mean value and standard error (Snedecor, 1946). Six cultures in each series.

not growth stimulating. Accordingly attention was directed in the main toward derivatives of ribonucleic acid. Assays were run with commercially available RNA (Schwartz), a mixture of four mononucleotides in equal parts, a similar mixture of nucleosides, and a group of free bases. Table 2 summarizes the results of these experiments. It is apparent that none of the substances tested produced areal increases over the unsupplemented controls, with inhibition apparent at high levels of supplementation. Microscopic observation likewise failed to reveal differences between experimental and control cultures.

Added co-factors.—Supplementation of the basal dialyzed media with a variety of known accessory factors failed to duplicate the

effects of crude dialysate. A selected group of data obtained in assays are shown in Table 3. At the concentrations employed adenosine triphosphate, fructose diphosphate, diphosphopyridine nucleotide and cytochrome C were all inert both singly and in combination.

TABLE 3

EFFECT OF ADDED CO-FACTORS ON OUTGROWTH OF CHICK HEART FIBROBLASTS IN DIALYZED MEDIA*

Supplement	Concentration in medium	Areal increase† at 10 days, mm^2
Basal medium (no supplement)	———	48.5±2.4
Basal + ATP (adenosine triphosphate)	0.001%	44.4±2.5
Basal + HDP (fructose diphosphate)	0.03%	45.2±2.2
Basal + DPN (diphosphopyridine nucleotide)	1 X 10^{-4} M.	40.4±2.2
Basal + cytochrome C	0.01%	36.2±3.5
Basal + ATP, HDP, DPN, cytochrome C	each as above	46.1±1.8

**Medium:* 0.5 ml. dialyzed chicken plasma + 40% dialyzed horse serum, 15% dialyzed EE_{50}, 45% Gey's solution.

‡Mean value and standard error (Snedecor, 1946). Six cultures in each series.

Microscopically the cell picture in supplemented cultures was uniformly similar to the vacuolated, refractile appearance of controls. A crude preparation of Coenzyme A was also assayed in serum cultures and in embryo extract-serum-saline but did not show signs of growth stimulation.

Brief mention may also be made of negative results obtained when dialyzed assay media were supplemented with Fischer's No. V-614 mixture (Fischer *et al.*, 1948), Medium No. 199 (Morgan, Morton, and Parker, 1950), and Simms ox serum ultrafiltrate (obtained from Microbiological Associates, Inc.). None of these materials when added at 10-25% levels in the medium produced changes in cell appearance or colonial pattern similar to those obtained with crude dialysate. Area increases with No. V-614 and No. 199 were slightly greater than controls when assayed in embryo extract-serum-saline at 0.085% $NaHCO_3$. When the buffer strength in subsequent assays was raised to 0.21% $NaHCO_3$, however, these differences were largely obliterated. Areas in cultures with serum ultrafiltrate were similar to those of controls. Supplementation with Protogen A at levels of 1, 10 and 100 units/ml. respectively was likewise without effect in assay cultures, both in dilute serum and in embryo extract-serum-saline.

Discussion

The significance of methods used in the present investigation to reveal the active agents in crude dialysate hinges on recognizing the principle of limiting factors. Thus the negative results obtained with supplementary amino acids and various co-factors do not imply that these substances are not essential for growth of the cells in question but merely that their existing concentrations in the unsupplemented assay medium may not be *limiting* for the deficiency syndrome produced by dialysis. It is apparent that dialyzed media are appropriate only for the assay of substances which remain at suboptimal levels in the control medium during the culture period. The active factors of the crude dialysate, whatever their exact nature, appear to fall in this category.

Further fractionations of the crude dialysate now in progress may lead to a more adequate characterization of the active factors. The results reported here suggest that the deficiencies produced by dialysis do not stem primarily from inadequate levels of amino acids or the lack of available energy sources, and that the active agents concerned are not accounted for on the basis of substances present in the most complete synthetic nutrients yet devised (e.g.—Morgan and Parker No. 199). On the other hand it is conceivable that the effects of crude dialysate may yet be duplicated by adding additional known substances to the present nutrients, altering the absolute levels of existing components, or changing their proportions. The possibility of unrecognized synergism between nutrient factors complicates the interpretation of assays made with individual supplements.

Similar difficulties may underlie a basic difference between the present experiments and the earlier investigations of Fischer and co-workers (see summaries in Fischer, 1941, 1946, 1948). Using chick fibroblasts and media prepared by dialysis with Ringer-glucose solution these authors consistently observed growth stimulation when cystine was added to the assay medium, with complete failure of outgrowth in its absence. Although this failure of outgrowth as such apparently reflects inadequate control of pH and buffer capacity (Harris, 1952) we have been able to confirm a stimulating effect of cystine supplements on the appearance of cells and explant area in Ringer-dialyzed media. On the other hand, when pH levels and buffer capacity are stabilized during dialysis as in the present experiments, the cystine effect disappears. Using media dialyzed against

F-2 solution we have consistently found that a basal outgrowth develops without supplementation, while the addition of cystine causes reduction of surface area with increasing vacuolation and refractility in the outgrowth zone. The basis for the cystine effect reported earlier by Fischer thus remains as an interesting problem.

Acknowledgment

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Summary

1. The growth-promoting effect of dialyzable factors in 12-day chick embryos has been investigated, using dialyzed media as a basis for assay. Test fractions were obtained from fresh embryos by extraction and dialysis in 70% ethanol. The alcoholic dialysates were collected and evaporated over $CaCl_2$, and subsequently were reconstituted in physiological saline for study in culture.

2. The crude dialysate increases surface area and cell number in the outgrowth zone of test cultures, and corrects a characteristic deficiency syndrome of cell appearance and colonial pattern in the unsupplemented assay medium. A correlated rise in glycolysis is also evident from determinations of lactic acid in supernatant culture fluids.

3. The active agents in the crude dialysate are non-protein, insoluble in lipid solvents, partially heat-stable but are destroyed by acid hydrolysis.

4. The effects of dialysate fractions were not duplicated by supplementing the assay medium with a balanced mixture of 20 amino acids, by ribonucleic acid or its cleavage products, or by several cofactors tested separately and in combination. Addition of ox serum ultrafiltrate (Simms) as well as complex synthetic nutrients (Fischer No. V-614, Morgan and Parker No. 199) likewise failed to produce the characteristic changes noted with dialysate.

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A THEORY OF DIFFERENTIATION

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INTRODUCTION

Several years ago the present author proposed a theory of growth limitation that combined bio-electrical and anatomical facts in a new way (Moment, 1946). Since then substantial confirmatory evidence has been obtained, both anatomical (Moment, 1949a, 1950a, 1952a, Siess and Stegman, 1950) and physiological (Moment, 1949b, 1950b, 1952b, Reed, 1949, Van Fleet, 1949). However, the implications of this theory for the problem of cellular differentiation have never been explicitly set forth or explored. It is the purpose of this paper to make such an exploration and to consider the pertinent evidence.

THE SELF-PRODUCED INHIBITORY VOLTAGE THEORY OF GROWTH LIMITATION

The theory of differentiation to be presented here is a corollary of the original theory of growth limitation. That theory grew out of a reinvestigation, on a scale never before attempted, of posterior regeneration in earthworms. The reinvestigation showed, for the first time, that the number of segments regenerated posteriorly (from any level of the worm from which posterior regeneration is possible) is a linear function of the distance, in number of segments, of the amputation from the anterior end of the worm. Every time the level of amputation is moved 10 segments posteriorly, the number of segments regenerated declines by 10. In other words, new segments continue to be proliferated at the posterior regenerating tip until the large old segments plus the small new ones summate, on the average, to the species mean of approximately 90. Consequently 50 large old segments plus 40 small new ones have the same inhibitory value at the extreme posterior growing end as do 80 large old seg-

ments and only 10 small new ones. These facts led to the conclusion (Moment, 1946) that whatever is the nature of the force or influence that finally stops proliferation of new segments at the distal end of the worm, it must fulfill certain conditions. (1) It must be independent of the size of the units producing it. (2) It must be closely dependent on the number of those units in linear series. (3) Its action must attain its full force at the end of the series. The only known force that fulfills these three requirements is the e.m.f. of a series of Galvanic cells. In addition, it has been repeatedly demonstrated that electrical potentials can inhibit and control growth and differentiation (Lund, 1921, 1947, Marsh and Beams, 1952).

A consideration of all of these facts led to the theory which may be briefly stated as follows. In their linear dimensions animals continue to grow by the proliferation of voltage-producing units until, by the summation of these units, a critical inhibitory voltage is built up. The proliferation of these units, whether they be segments or cells, occurs in distal growing regions, and it is in such distal regions that the inhibitory voltage finally attains a threshold value. It should be noted that subsequent growth of the segments and of their constituent cells may be very great, but this growth is by cell enlargement rather than proliferation. For a detailed discussion of the relation of this theory to other growth regulating mechanisms and to different types of cells, the reader must refer to the papers cited above.

A Proposed Biochemical Mechanism

Previous work has shown that after a worm is cut in half, the voltage measurable end to end falls precipitously. During subsequent regeneration the restoration of the voltage to its original value and of the segments to their original number are parallel processes both reaching an asymptote at about the same time (Moment, 1949b). Furthermore, as the level of proliferation becomes more and more distal, not only does the voltage increase but *pari passu* the rate of regeneration becomes slower. Such a progressive retardation of growth as the level of regeneration becomes more distal was apparently first observed by Spallanzani in the limbs of salamanders but it has since been found to be a common and probably universal feature of regeneration and has been called "Morgan's law of regeneration" by Mayer (1910) and others.

This parallelism between the fall in the rate of regeneration and the rise in voltage at progressively more distal anatomical levels suggests a biochemical mechanism along the following lines. With the exception of one problematical case, in all plants (Lund, 1929, 1947), invertebrates (Barth, 1940, Moment, 1950b. Watanabé, 1928), and vertebrates (Burr and Hovland, 1937) tested, a growing distal end is electro-positive to more proximal regions. As regeneration proceeds, the growing end becomes increasingly positive with respect to the rest of the animal. This means that it becomes a region of relatively fewer and fewer electrons, or in other words, a region of increasing oxidizing potential. These facts seem to indicate that proliferation slows down and finally stops because conditions become increasingly favorable for oxidations. What is the evidence?

So many data have been published indicating that reducing compounds, and conditions favorable to reduction are either associated with or actively stimulate proliferation that it is possible to mention only some of the more noteworthy here. Hammett (1929) was one of the first, if not the first, to discover that reducing compounds of the sulfhydryl group were abundant in the proliferating zone of root tips, that heavy metals which will form mercaptides with the –SH radical would inhibit cell division but (significantly for the present theory) not cell enlargement, and that sulfhydryl compounds, especially glutathione, would stimulate cell division in both root tips and Paramecium.

Hammett's work was at first subject to adverse criticism by Morgulis and Green (1931) among others. However, the stimulative effect of sulfhydryl compounds on cell division was confirmed by Voegtlin and Chalkley (1930) and Chalkley (1951) in a series of investigations on Amoeba, by Coldwater (1933) on Tubifex, by Baker (1929) and others on fibroblasts *in vitro*, and by Jahn (1934) on Chilomonas. At the same time Rapkine (1930, 1938) and his associates amassed much additional evidence of a similar sort, and developed an elaborate theory about the relationship between sulfhydryl compounds and cell division in terms of the reversible denaturation of proteins. Brachet (1950) today regards Rapkine's hypothesis as the best so far proposed to account for all the biochemical and cytological facts known about mitosis.

Hammett and his associates (Hammett and Chapman, 1930, Hammett and Hammett, 1933) in a long series of papers presented many

of the details of the sulfhydryl effect and demonstrated on a variety of organisms that –SH compounds stimulate (or permit) cell division but do not promote either cell enlargement or differentiation.

Approaching this problem from another side, Reed (1947, 1949) has recently shown that if the oxidation-reduction potential in plant meristems shifts so that the naturally occurring dihydroxyphenols become oxidized to quinones, mitosis is blocked. If suitable hydrogen donors, such as sulfhydryl compounds, are added in amounts sufficient to restore a reducing potential, cell division will begin again. Van Fleet (1949) has demonstrated such changes in plant tissues cytologically, and found that extractable anti-oxidants are abundant in proliferating, non-differentiating regions, while pro-oxidants are abundant in regions where cell division has stopped and differentiation is proceeding.

In intact annelids and other animals, Hyman (1940) and Child (1941) have repeatedly found a U-shaped gradient of respiration, i.e., respiratory activity increases as either end of the animal is approached. Such a progressive increase in respiration in more distal regions, obviously enough, harmonizes both with the decline in rate of regeneration at more distal levels (Morgan's law) and with the facts just cited about pro- and anti-oxidants in plant tissues as well as with the general theory under discussion here.

Concerning the Theory of Apical Dominance and Axial Gradients

A consideration of the facts presented in the preceding sections makes it apparent that a theory of growth limitation such as that presented here has important implications for any theory of differentiation, and especially for the theory of axial gradients as it applies to differentiation. It may be that the inhibitory voltage not only sets the perimeters of the animal in the sense that it establishes the maximum size, the outside dimensions. The self-produced voltage may also determine what might be called perimeters of differentiation. What is the evidence for this?

Ever since the work of Fischer and Parker (1929), it has been known that there is an antagonism between proliferation and differentiation. It is now an accepted rule of tissue culture that "growth necessitates frequent renewal of the culture medium while differentia-

tion, on the other hand, requires avoidance of disturbance of the tissues and a limitation of growth stimulating substances" (Cameron, 1950)

This antagonism between proliferation and differentiation, together with the parallelism between the decreasing rate of regeneration and the increasing voltage and respiration in more distal regions, suggests a reinterpretation of Child's theory (1941) of apical dominance and axial gradients in the development of new hydranths in coelenterates and similar phenomena in other organisms. It will be recalled that, according to Child, a stolon grows out from a hydranth as an undifferentiated structure until it "escapes" from the "dominance" of the original, apical hydranth. When the proliferating end of the stolon has grown out far enough to have "freed" itself from the dominance of the original polyp, a new hydranth is able to differentiate. The physical meaning to be attached to the word "dominance" is left extremely vague, except that dominance is held to be "transmissive" rather than "transportive" in character.

According to the self-produced inhibitory voltage theory of growth limitation, a new hydranth forms when and where it does, not because a stolon has grown out so far that its distal end has escaped from the influence of a dominant apical region, but, on the contrary, a new hydranth differentiates when and where it does precisely because the growing end of the stolon is approaching the end of its rope. Proliferation has been inhibited to an extent sufficient to permit differentiation to set in.

As noted above, the explanation offered by the theory proposed here squares with the facts of Morgan's law. Regeneration is progressively slower in more distal regions. This would seem to be the exact opposite of expectation if the growing end of the stolon were gradually escaping from some inhibitory influence.

Thus the present theory of growth limitation and differentiation can be regarded as a development of the theory of axial dominance in the direction of a closer fit to all the facts, as well as of greater physical and biochemical definiteness. Dominance is no longer conceived of as resident in a special center from which it extends by some unknown process and in decreasing intensity. On the contrary, in segmented animals, all the segments may contribute equally to the terminal inhibition. At the same time there will be a gradient of electrical potential and, correlated with it, a gradient of respiration.

Since the controlling influence is held to be electrical, it is presumably "transmissive" in the sense of Child. And since in the present theory, the differentiation of a new individual occurs because a critical level of inhibition has been reached, rather than because the proliferating end has escaped from an apical dominance, the present theory may perhaps be called the theory of Child in reverse, with a more definite biochemical meaning added.

Various investigators, including Child (1941), have attempted to interpret embryonic organizers in terms of axial gradients and dominance. No specific attempt will be made here to interpret organizers in terms of the present theory. However, it should be remembered that Berrill (1931) and Berrill and Mees (1936) after an extensive investigation of regeneration and differentiation in a polychaete were unable to explain their results either on the basis of a diffusing organizer substance, or of dominance and axial gradients. This difficulty was first pointed out and emphasized by Gross and Huxley (1935) on the basis of their own and Berrill's work. In the end, Berrill and Mees suggested that some kind of electrical forces seemed to be the most probable explanation for the observed facts of morphogenesis in these regenerating polychaetes.

Concerning Specific Differentiations

All these facts suggest a further question. Is the specific type of cellular differentiation controlled, at least in part, by the degree of inhibition? Whether causally related or not, in regeneration in salamanders, the high degree of inhibition and slow proliferation at the wrist is correlated with the differentiation of a hand, and the relative lack of inhibition and rapid growth at the shoulder with the formation of an upper arm. In the embryonic chicks, Saunders (1948) has shown that the sequence of development of the parts is similar to that of the limb regeneration just mentioned. In his own words, "the materials for the future wing parts are laid down in proximo-distal order and in their definitive spatial pattern, apparently by a specialized growth zone in the apical region of the bud". Dalcq (1949) has described the differentiation of a vertebrate limb along its proximodistal axis in terms of nutritive competition among its parts, after the manner of Spiegelman (1945), and has tentatively suggested "that limb formation and regeneration should perhaps be considered under the unusual aspect of some basal dominance being

released and, correlatively, more and more centers of competition becoming active in more distal territories".

The essential vagueness as well as some of the difficulties inherent in theories based on the concept of "dominance" and "escape" have been discussed above. On the other hand, the fact of nutritive competition is well established. A starving salamander will regenerate a new tail or a new limb even after repeated amputations (Morgan, 1901). An earthworm without food will regenerate the "correct" number of segments to correspond with the level of amputation (Moment, 1952b). In such cases the regenerating tissue must grow at the expense of the rest of the organism. But when the number of tail vertebrae or of posterior segments characteristic of the species has been attained, proliferation stops. It cannot be because food reserves have been exhausted as the ability for repeated regenerations during starvation proves. Furthermore, the more posterior is the level of amputation in an earthworm, the slower is the rate of regeneration and the fewer the number of segments formed, even in a starving worm. This would seem to be the reverse of expectation if the amount of available food were the controlling factor. Nevertheless, as Liebmann (1942, 1945) has shown, a short piece of worm with relatively small food reserves will regenerate faster and produce more new tissue than a longer piece of worm with far more food reserves, both as specialized storage tissue (trephocytes) and as developed muscles and other structures. This result of Liebmann has been repeatedly confirmed in this laboratory. On the basis of a nutritive theory it remains a paradox.

Yet Morgan's law appears to hold good throughout the animal kingdom. Some factor or factors evidently control the rate at which proliferating cells at a growing tip lose their competitive advantage. Consequently we return again to the theory that a gradually increasing inhibitory voltage provides a graduated regulatory mechanism that would set the level of competitive interaction. If different degrees of inhibition favored different specific differentiations, then many remarkable facts of animal structure would receive an explanation. For example, many segmental animals such as the annelids and crustaceans, possess some beautifully precise mechanism that accurately places certain organs in certain segments. There may be ovaries within the 13th, feathery gills from the fourth and fifth, or specialized maxillipeds on the eighth. An increasingly inhibitory

voltage that is closely correlated with the linear position of the segment in the animal would provide the kind of regulatory mechanism that appears to be present. As in earthworm regeneration, it would enable the animal, as it were, to count its segments.

On the biochemical level, the hypothesis that the type of differentiation depends upon the degree of inhibition presupposes that different metabolic pathways are favored by different degrees of inhibition, i.e., different oxidation-reduction potentials as indicated in a previous section. Different metabolic pathways with different enzyme systems active, in turn lead to different end-products, in other words, to differentiation. The work of Clark (1928), Michaelis (1933), and others have made it evident that biological substances like enzymes, coenzymes, and enzyme-substrate complexes occupy different positions on the scale of oxidation-reduction potentials. Consequently there will be a regular sequence in which they are influenced by a changing electrical potential. Certain systems will be inhibited before others. The results of such sequential inhibition can be expected to be especially significant when more than one enzyme system is competing for the same substrate.

Of especial importance for the present theory is the modern knowledge of the sulfhydryl groups on which Hammett and Rapkine worked. Hellerman, himself a pioneer among chemists in this field, has recently emphasized (1947) the key rôle which –SH radicals play in many important biological oxidations including those involving pyridine nucleotides and other protein-related reactions as well as the strategically placed pyruvate molecule. In this latter connection it is important to note that Bullough (1952) and his associates have amassed an impressive amount of evidence that the rate of cell division is directly related with the Krebs cycle and mentions the sulfhydryl enzymes specifically.

In the living cell, Barron (1952) has pointed out that the *actual* metabolic pathways taken are determined by the multiple regulatory mechanisms of the cell. He lists the topographic distribution of enzymes, pH, hormones, electrolyte distribution, and the oxidizing potential. The present theory merely points to the effect that bioelectric potentials, which are known to be present, can reasonably be expected to have on cellular oxidation-reduction potentials. Unfortunately little is known about the optimum oxidation-reduction potentials for the various enzyme systems, although those which have been studied show a

definite optimum on either side of which activity drops off asymptotically (Barron, 1951).

There is indeed both old and new evidence for the interrelationship of sulfhydryl compounds and oxidation-reduction potentials. As long ago as 1934 Jahn (*ibid.*) presented facts to show that –SH compounds exert their stimulating effect on cell division through an effect on oxidation-reduction potentials. And recently Maver and Thompson (1946) after a long study of the rôle of –SH groups in protein-active enzymes came to the conclusion that these potentials are the most effective of all the factors controlling the activity of the –SH enzymes. Within the last few months Lallier (1952) has presented evidence indicating that the activity of the animal-vegetal axis of the echinoderm egg is controlled by the oxidation-reduction potential of the cells. According to this view the "animalizing" and "vegetalizing" agents and procedures discovered and so extensively investigated by the Scandinavian school of embryologists, function by virtue of their effects on these potentials. This conclusion agrees with the ideas of Yamada (1950) to the extent that the latter worker regards the dorso-ventral axis to be qualitatively the same throughout.

In conclusion, the general theory of differentiation here proposed and its various ancillary parts should be considered as a development of the field theory of morphogenesis in the direction of greater definiteness. In the words of Huxley and DeBeer (1934), after summarizing a great mass of facts favoring a field theory, "the fate of the regenerated tissue is determined in relation to the *level* of the gradient at which regeneration is made to occur, not to the specific tissues present at the cut surface". And Weiss, himself one of the originators of the field theory, in a recent paper on morphogenesis (1950), has suggested that "field effects" are due to "transmission rather than convection. That is to say, control over large cellular collectives may not spread by substances being bodily transplanted from cell to cell." The present theory, while in no sense denying the importance of nutritive and hormonal factors, supplements such factors with an overall mechanism of control that possesses both a specific physical and biochemical meaning and a spatial, i.e., morphological, dimension.

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EFFECTS OF INTENSE ULTRASONIC VIBRATIONS ON *PISUM*. I. EFFECTS ON ROOT MERISTEMS[1]

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INTRODUCTION

A number of investigations have been concerned with functional and structural aberrations of higher plants induced by ultrasonic vibrations. Ultrasound has been observed to cause nuclear alterations, chromatid and chromosome breaks, vacuolization of nuclei, chromosomes and cells, and considerable necrosis (6, 7, 11, 12).

Most biologic investigations demand ultrasound of very high intensities. Energy of this sort exhibits thermal effects and oxidizing effects, strong dispersive power, a rapid acceleration of particles, and a formation of gas bubbles by cavitation (4, 8). Cavitation is the presence of hollows or cavities in the liquid formed by the tremendous pressure differences existing between the crest and trough of the ultrasound wave. Considering that particle acceleration may be above 10^5 times greater than that of gravity, it is reasonable to expect that biologic systems and chemical reactions would be affected by intense ultrasound.

Newcomer and Wallace (6) reported that the immediate effects of ultrasonic vibrations on plant cells are similar in many respects to the primary and secondary effects noted for electromagnetic waves and for mustard gas. They also postulated an interference of the nucleic acid cycle of the nucleus due to spatial mixing of cellular components. Loza (5) in his investigations on pea roots found a direct relationship between effects and thickness of root and he also observed a differential resistance for tissues exposed to ultrasonic energy.

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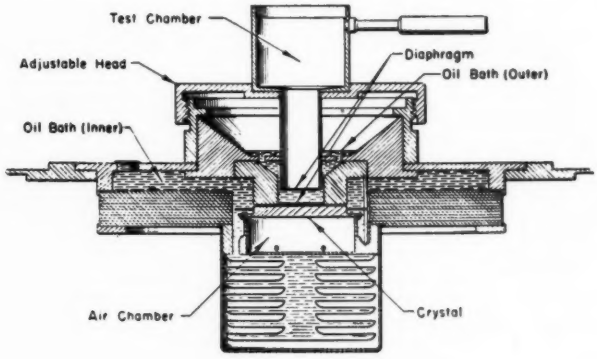
Conger (1), using *Tradescentia* buds found that sonic energy applied simultaneously with X-rays increased chromosomal aberrations, but that sound alone did not produce disturbances. It is of significance to note that Stumpf *et al.* (10) concluded that degree of cellular disintegration is independent of frequency and dependent only on the intensity and on the duration of exposure.

To summarize, it appears that ultrasonic vibrations induce structural aberrations in plant material and that these effects are similar to those produced by other types of radiation and by chemical means. Although certain cytological and gross morphological disturbances have been studied in detail, there is little information concerning plant tissue responses. The present study has been undertaken to examine more closely the nature of the ultrasonic action on certain aspects of the histology of root apices.

Materials and Methods

The ultrasonic apparatus was developed by Raytheon Manufacturing Company to serve as a high frequency vibrator for liquids. This vibrator consists of an electrically driven quartz crystal ground to a thickness of 5.7 mm. and resonant at 500 kilocycles per second. The bottom face of the crystal is goldplated and is mounted in contact with the air, while the upper face is immersed in an oil bath about .25 mm. inch below a thin Monel Metal diaphragm (upper electrode)

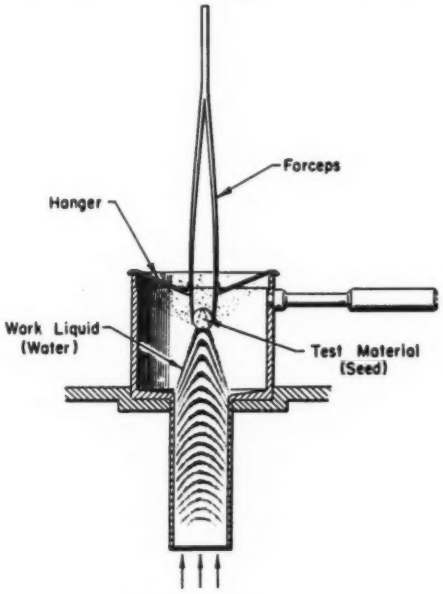


Ultrasonic Vibrator.
FIGURE 1

(Figure 1). Above this diaphragm is an outer oil bath in which the container holding test materials is immersed. A vacuum tube oscillator furnishes the necessary power to the vibrator.

The power output from the crystal was adjusted to one of two values, 20 watts per cm.^{2} (A), or 10 watts per cm.^{2} (B), for all of the treatments in the course of this investigation. The output was computed calorimetrically and a barium titanate transducer, placed always in the same geometrical location was employed to insure uniformity of radiation pressure for the duration of these experiments.

Living, intact root tips of pea (*Pisum sativum* L.) were exposed by holding them in the apex of the cone created by radiation pressure in the surface of the distilled water within the chamber. The thin, flat bottom of the container was immersed in the oil bath so that the water (17 cc.) in the lower stem of the chamber was subjected to substantially the same intensity as if it were directly in contact with the crystal. The temperature of the water was main-



Test Chamber.
FIGURE 2

tained below 30° C. and usually near 25° C. by cooling and by changing the chambers. The root tips, made less flexible by glass collars 2 cm. in length through which 1½ cm. of the root apex protruded, were held rigidly in place by forceps anchored to the upper rim of the chamber (Figure 2).

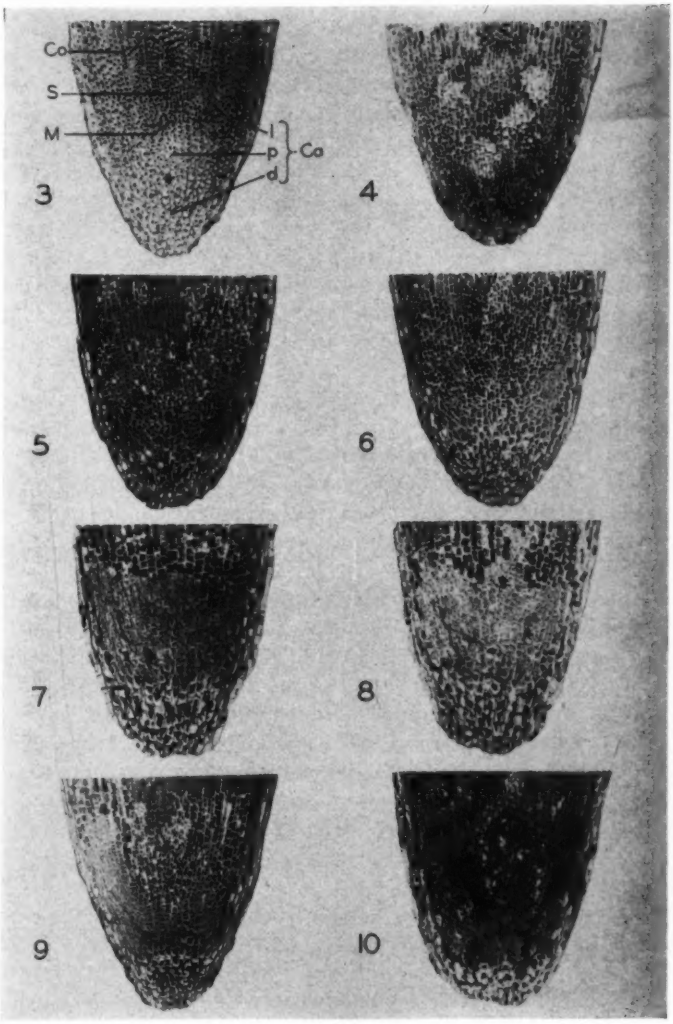
The root apices were primarily divided into two lots, one lot being subjected to the higher intensity (A) and the other to the lower intensity (B). These categories were secondarily subdivided into 4 groups according to the duration of exposure: I, 10 secs.; II, 15 secs.; III, 20 secs.; IV, 30 secs. (Treatments of 60 seconds duration were also performed but their invariable lethality adds little to the data.) All data recorded are for Woodruff's "Little Marvel", a line of highly inbred, homozygous peas. A second variety, Burpee's "Laxton", showed that the effects observed for ultrasonic treatment of "Little Marvel" root apices were not unique.

The root tips were from 10-day-old plants of the "Little Marvel" line that had been grown in moist sand at about 25° C. These tips were treated intact and then either the apical 1 cm. was severed and immediately killed and fixed in Randolph's modification of Navashin's fluid, or the supporting collars were removed and the seedlings replanted for specified periods (24, 48, or 72 hours). Fixed material was sectioned by the paraffin method and stained by the Feulgen reaction, Heidenhein's haematoxylin, or Pappenheimer's modification of Unna's methyl-green pyronin mixture.

Results

The effects of ultrasonic vibrations upon the histological and cytological organization of root apices were correlated with both duration and intensity of exposure. For simplicity, and since pea root apices lack discernible "histogens", areas of root tips are discussed in terms of meristem (Figure 3, M) cortex (Figure 3, Co), stele (Figure 3, S) and root cap or calyptra (Figure 3, Ca). In the latter structure three general regions are designated: distal (Figure 3, d), proximal (Figure 3, p) and lateral (Figure 3, l).

The meristem, as here employed, is equivalent to the area sometimes termed promeristem and gives rise to root cap and to root proper as a result of its activity and position. For reason of the gradual transition between cells of the meristem and cells of adjacent regions, the meristem can be delimited only in an arbitrary sense.



FIGURES 3-10
EXPLANATIONS IN TEXT

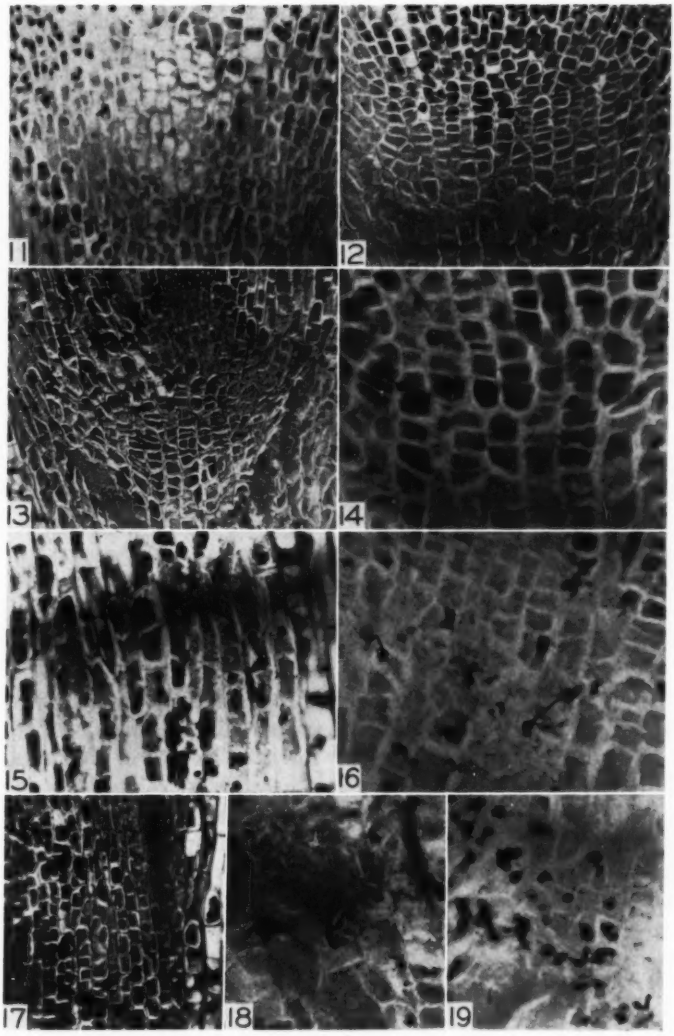
Patterns of observable disturbances have a sequential development which increases in severity with time and with intensity of treatment. At either energy level, A or B, the initial agitations effected nuclear alterations. Further agitation aggravated this situation until entire protoplasts were involved. Finally, complete loss of cellular organization resulted, even to disintegration of cell walls.

Energy level B produced no detectable disturbances in root tips until the duration of exposure was extended to 15 seconds at which time the proximal portion of the root caps showed some nuclear aberrations. Treatment for 20 seconds at the same intensity gave rise to additional disturbed areas in the cap and cortex (Figure 4) and also accentuated the nuclear and chromosomal disorder noted for the shorter exposure. In fact, in sections and smears stained by any one of several methods, the centers of affected areas revealed cells completely devoid of nuclear material. A majority of cells around the periphery of these areas were apparently undisturbed, the greater proportion of nuclear disruptions and abnormal configurations were located between the center and periphery (Figure 11). These areas, therefore, have the appearance of vibrational foci in that the intensity of the vibrations apparently diminishes with the distance from the center.

Root tips subjected to 30 seconds exposure showed an enlargement and coalescence of affected areas.

The same sequence of effects as brought about by intensity level B was observed in sections of root tips treated at the higher level, A. However, nuclear irregularities were more frequent and more pronounced, and disintegration of cell walls more general. In longitudinal section the affected regions did not have the circular outline so characteristic of the lower power exposure, instead the areas of apparently greatest turbulence were elongated and occurred parallel to, and often contiguous with, boundaries between regions of the root tip.

The first effects of more intense energetic vibrations were manifest in the lateral portions of the root cap when the duration of treatment was extended from 5 to 10 seconds (Figure 5). A further increment to 15 seconds seemingly did not cause a corresponding increase in the disruption of cell walls, but when the sections were stained for chromatin material a greater loss of chromaticity in the centers of affected areas and more numerous nuclear aberrations around the periphery were seen (Figure 6). Agitation of root tips for 20 seconds



FIGURES 11-19
EXPLANATIONS IN TEXT

provoked a more general disintegration of cell structure in that the destruction noted for the 15-second exposure spread basipetally until ultimately the cells beneath the meristem, in the proximal region of the root cap, became involved. In longitudinal section the area of greatest apparent modification formed a U-shaped mass. The base of the U was located in the proximal portion of the cap while the arms projected into the lateral extensions of the cap (Figure 9).

The nuclei of the meristem were greatly distorted and the cell walls of the adjacent calyptra were almost completely disrupted (Figure 12). The chromatin material of two or more cells often aggregated, some of the material apparently passing through intact cell walls (Figure 14). Occasionally, these chromatin strands, linking a number of clumps of the same material as well as other nuclear masses, were detected in areas where cell walls had been largely destroyed (Figure 16).

Subjection of root apices to vibrational forces for 30 seconds resulted in an almost complete breakdown of tissues involving not only a large portion of the calyptra but also the meristem and adjacent regions of the stele and cortex (Figure 10). Again, the lateral portions of the root cap were more severely affected than were other regions of the root. Around the perimeter of these sectioned, heart-shaped masses of disorganized cells a number of very prominent aggregations of chromatin material was evident (Figure 13).
dent (Figure 13).

Root tips that were not killed and fixed until 48 hours after 15-second treatments revealed, in sections, development of necrotic areas which included most of the internal structure of the apices (Figure 7). A similar pattern of necrosis developed in apices subjected to ultrasound for 20 seconds but not killed and fixed until 24 hours after exposure (Figure 8). Inspection of tips killed and fixed after this same lapse of time but treated for an additional 10 seconds (30 sec. total) revealed that although the dosage is lethal to the same tissues as were the 15- and 20-second subjections, the affected regions are more extensive.

The cells of the cortical region (killed and fixed immediately following exposure) usually demonstrated the same sort of disturbances noted for other regions (Figure 15). However, this same region often showed a number of roughly spherical, deep staining

granules in each cell when the root tips were killed and fixed after an interval of twenty-four hours (Figure 17).

An apparently violent dispersal resulting in a diffuse arrangement was often noted for the chromatin material in the distal portions of root caps that had been severely agitated and then killed and fixed immediately (Figure 18). This effect all but disappeared in material that was not killed and fixed until fifteen minutes after exposure (Figure 19).

An average of approximately one in five root tips that recovered from an exposure of 20 seconds duration at energy level A showed a marked increase in growth over that observed for control roots. This response was not immediate; however, the difference in development could be detected within a week after treatment.

Discussion

The effects of ultrasonic energy upon the cells of root tips of pea were substantially the same sort of disturbances described by other investigators (5, 6, 12). Such effects included changes in nuclear form, aggregation or clotting of chromatin material, disruption and vacuolization of nuclei, apparent loss of chromaticity, and necrosis. Differential resistance for tissues of root apices has been noted by other workers. Loza (5) concluded that the effects were proportional to the thickness of the tissue; therefore, the central regions or meristem of the root tip would be least affected. However, in light of the similarity between the observations reported here and the work of Newcomer and Wallace (6) this differential effect is probably more correctly attributed to the denser cytoplasm of the nearly isodiametric cells of the meristem which provides a much more rigid matrix for the nuclei and chromosomes. The most sensitive regions were those tissues composed of cells having a high degree of vacuolization and thin primary walls. The large vacuoles present in the calyptral cells probably cause this tissue to exhibit the more profound alterations such as loss of chromaticity, collapse of cell walls, and necrosis. According to Harvey and Loomis (3) considerably more power is required to produce cavitation in heavy, viscous liquids, *e.g.*, cytoplasm, than in the lighter liquids of vacuoles. This is explained by the fact that viscous liquids have a higher cohesive pressure. Sigenaga (9) concluded from his extensive studies that vacuolization in the cytoplasm is not a necessary factor for abnormal nuclear divisions but

that an impairment of the viscosity regulating power of the cell may allow other factors to influence division. Frenzel and his associates (2) demonstrated that ultrasonic energy greatly enhanced and accelerated diffusion through membranes.

A postulate concerning the variations in patterns cannot be invoked with certainty in view of the known physical properties of the radiation although the histological responses to the two levels of ultrasonic energy, 20 watts per cm.^{2} and 10 watts per cm.^{2}, were markedly different, especially in regard to those regions which were first affected. The employment of a constant wave length in this investigation allows the differential responses to be more reasonably accepted if either dissimilarities in the biologic systems of the areas or a greater tendency toward a physical separation of calyptra from the root apex proper, attributable to the more powerful level of exposure, is assumed.

The effects of ultrasonic radiation on living material are dependent only on intensity when time is constant. This is also a characteristic of cavitation. Stumpf and his associates (10) found that when power was raised beyond a certain point, denaturation and inactivation of the labile proteins took place in a rather narrow range and that some types of cells were easily disintegrated whereas others were completely refractory. The cells of various regions of the pea root apices may manifest different responses because of variations in their components and a reactive correlation between these variants and selective intensities.

The patterns of disturbances provoked by the higher level of energy suggest that these vibrational forces tend to cause a separation of the calyptral regions from the meristematic zone. The lower level of power was insufficient to sever these tissues even though capable of causing considerable cytological disorder. The initial impact of the more forceful vibrations may well have weakened the delicate cell walls of the interior of the lateral portion of the root cap while further exposure made this debility manifest as completely ruptured walls. In time the disruption of cells spread basipetally until the root cap was almost completely parted from the meristem. It may be significant that this tissue which was first to break down is characterized by possessing cells having thin walls and a high degree of vacuolization. Few cell walls were disrupted in the adjacent meristematic zone until the dosage was of sufficient duration to involve nearly all the other apical tissues. The meristematic cells showed a wide

variety of nuclear conditions as would be expected for tissues with nuclei in mitotic phases, yet their relatively thin walls apparently were able to withstand the tremendous vibrational forces. Perhaps the durability of the meristem is due to the very low degree of vacuolization so typical of karyokinetic regions.

Observations on the effects of intense ultrasonic vibrations on pea root apices suggested that this kind of energy may be employed to induce morphogenetic change. The ability of the meristem to withstand disorganization and cell wall disruption even though its nuclei showed extreme aberrations may represent an ultrasonic effect useful in radiation cytology. Secondly, the resumption in growth noted for treated root tips, some of which were exposed to the higher level of power for as long as 20 seconds, was probably based on the durability of the meristem and may serve as a motive for various ontogenetic studies. The marked increase in length of a few treated roots over their controls arouses certain speculation as to the nature of this and other developmental aberrations. Finally, the distinct cellular alterations, *e.g.*, loss of chromaticity, etc., may be linked to fundamental shifts in the physico-chemical systems of the cell.

Summary

The effects of intense ultrasonic vibrations on root apices of *Pisum sativum* L. were studied. The frequency employed was 500 kilocycles per second and the powers developed were 10 watts per cm.^{2} and 20 watts per cm.^{2}.

Agitation by ultrasound induced cytological and histological aberrations in the root tips. These disturbances increased with length of exposure. The initial exposures caused nuclear disorders, while further treatment disorganized entire protoplasts. Finally, sustained dosage resulted in complete loss of cellular arrangement, loss of chromaticity, disintegration of cell walls, separation of tissues, and necrosis.

The nuclei of the meristem displayed the greatest numbers and kinds of aberrations but the calyptral regions showed a higher degree of cellular disorder and cell wall disruption than did the other tissues of the root. In general those areas characterized by a high degree of vacuolization manifested the severest disturbances.

The higher level of power, 20 watts per cm.^{2}, promoted a separation of the calyptra from the meristem, while the lower power, 10 watts per cm.^{2}, caused little tissue disjunction.

The use of this medium of energy to induce morphogenetic change is suggested from observations which include: 1, the ability of the apical meristem to withstand histological disorganization even though its nuclei showed extreme aberrations; 2, the recovery of treated root tips; 3, a marked increase in length over controls for some of the treated roots; and 4, the apparent physico-chemical changes.

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EFFECTS OF INTENSE ULTRASONIC VIBRATIONS ON *PISUM*. II. EFFECTS ON GROWTH AND THEIR INHERITANCE[1]

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INTRODUCTION

Part I of this study (20) described the effects of intense ultrasonic vibrations on the root apices of *Pisum sativum* L. Among the consequences of treatment was a decided increase in length over their controls for several exposed roots. Part II is devoted to (a) an attempt to induce the same sort of effects in shoot apices of *P. sativum*, (b) a study of the inheritance of such modifications as arise, and (c) certain physico-chemical analyses of treated material.

Stimulated growth, earlier maturation, increased yield, and cytogenetic variations have been attributed at one time or another, to ultrasonic treatment. Other investigations have shown inhibitory and injurious effects. Istomina and Ostrovsky (10, 11) noted early maturity and increased yields for peas and potato tubers subjected to ultrasonic waves before planting. Newcomer and Wallace (16) found that resumption of growth in root tips of *Narcissus* following ultrasonic treatment was often accompanied by a marked stimulation, and they also noted early maturation of other (unnamed) plants after exposing their apical meristems to ultrasound. Loza (14) concluded that root development is retarded or arrested by high frequency sound waves and that seeds seem to be insensible to these radiations. A dosage which promoted an increase in growth of seedlings was shown by Ollivier (17) to be inhibitory when applied for a longer duration.

Statements in the American lay press (2) reported yields of hybrid

[1]Contribution No. 862 from the Massachusetts Agricultural Experiment Station. The assistance and facilities provided by the staff of the Plant Research Institute, Austin, Texas, during a portion of this investigation are gratefully acknowledged.

maize increased fifteen per cent after treating the grain with ultrasound.

Statistical analyses made on earliness, height, tiller number, and number of leaves of maize by Haskell and Selman (8) showed no significant differences between plants arising from seed treated with ultrasound and untreated plants. Schwabe and Thornley (19) exposed Petkus winter rye seed to ultrasound and found a slight favorable effect on germination rate of the unsoaked seed although time to flowering was not affected and no vernalization resulted.

In an attempt to cause mutations in *Drosophila melanogaster* by means of ultrasonic radiation, Hersch and his associates (9) treated a male fly and crossed it with an untreated female. Although over 26,000 flies were raised from this mating and the evidence was inconclusive, the abnormalities that did arise in the population were unlike those induced by X-rays or radium. A high frequency of unilateral wing mutations in the F_2's of ultrasonized *Drosophila* has been noted (21).

Inheritance of ultrasonically induced variations in plants has not been reported; however, Wallace, Bushnell, and Newcomer (21) suggested that certain genetic changes in *Helianthus* are attributable to this kind of radiation.

Since exploratory experiments performed in these laboratories demonstrated an apparent stimulatory effect it was deemed advisable to study the incidence, nature, and basis for the introduced variation. Certain aspects of the physiology of ultrasonized peas were investigated in order to ascertain if there was any change in the biologic systems.

Materials and Methods

The seeds of *P. sativum* were treated in the same manner as were the root tips (20) except that glass collars were not employed and that the hilum was oriented to face the bottom of the exposure chamber.

Prior to treatment, seeds were soaked in three changes of distilled water at 25° C. for six hours and transferred to moist chambers for an additional 12 hours. Exposed seeds were either returned to moist chambers until analyzed or planted in four-inch clay pots. The plants were grown in a greenhouse or in controlled environment laboratories where light and humidity conditions were more easily

controlled. In these laboratories the temperature was maintained at 28° C. for daylight hours and 18° C. during darkness. During the duration of these experiments the temperature in the greenhouses varied from 7° C. to 33° C.

The treated generation and their progeny were compared with untreated peas as to several physical features, *e.g.*, dry weight, wet weight, height, internode length, time to flowering, etc. The exposed plants were self-bred since there would be less confusion between effects of ordinary F_2 gene segregations and effect of ultrasonic vibrations than if crosses were employed. The inbred treated and unexposed plants were crossed with control plants in an attempt to determine the nature and expression of induced aberrations. Additional generations of plants were grown from seeds derived from the progeny of exposed plants to see if the modifications encountered would diminish with time.

All physico-chemical analyses were made of seeds that had been exposed for 20 seconds at intensity level A (20 watts/cm.^{2}) or of plants arising from such seeds. Total nitrogen, total phosphorus, and free and hydrolyzed amino acid determinations were performed on treated seeds and on plants of different ages two generations removed from treatment.

Total nitrogen was determined on sulfuric acid-hydrogen peroxide digests of the dried, ground plant material by Nesslerization using the Koch-McMeekin (12) modification of Nessler's reagent. Total phosphorus was obtained by the procedure of Fiske and Subba Row (6). The Dent (5) method of detecting amino acids and other ninhydrin-reacting substances on filter paper chromatograms was employed, except that migration was accomplished by the scheme of Williams and Kirby (23).

Pea plans which arose from ultrasonically treated seed were also analyzed for crude fiber (3).

Total proteolytic activity of exposed, soaked seed was determined at various times before and after treatment. The analyses were based on the method of Mounfield (15) modified in that cysteine was used as activator, thymolsulfonthelein as indicator, and initial gelatin concentration was 1% instead of 2%. Activity is reported as increase in ml. N/20 NaOH per gram fresh weight per 5 ml. aliquot of digestion mixture after 24 hours digestion at 40° C.

TABLE 1

PERCENTAGE GERMINATION OF TREATED SEED AND LENGTH OF PRIMARY ROOT 15 DAYS AFTER TREATMENT

	Control		10 sec.		15 sec.		20 sec.		30 sec.	
Germinated	264	(88%)	235	(79%)	238	(56%)	110	(29%)	0	(0%)
Not germinated	36	(12%)	63	(21%)	184	(44%)	270	(71%)	286	(100%)
Primary root length										
Less than 5 cm.	35	(13%)	73	(31%)	150	(63%)	68	(61%)	———	
Five cm. to 10 cm.	229	(87%)	162	(69%)	83	(35%)	18	(17%)	———	
More than 10 cm.	0	(0%)	0	(0%)	5	(2%)	24	(22%)	———	

RESULTS

A. *Effects on seeds and on development of treated generation (T).*

Treated seeds showed two, distinct, primary effects. The percentage germination decreased from 28 per cent for the control to 9 per cent for seeds exposed 30 seconds at intensity level A (Table 1). Percentage germination for seeds treated for 10, 15, and 20 seconds was, respectively, 79, 56, and 29 per cent. In general, treatment depressed the elongation of roots; however, 22 per cent of the roots that arose from seeds exposed for 20 seconds, and 2 per cent from seeds exposed for 15 seconds, showed a noticeable increase in length over control roots at fifteen days (Table 1). In fact, these elongated roots had a length of over 10 cm. whereas 61 per cent of the seed that remained viable after similar treatment failed to produce primary roots over 5 cm. long. The remaining seedlings, 17 per cent, had primary roots between 5 cm. and 10 cm. long.

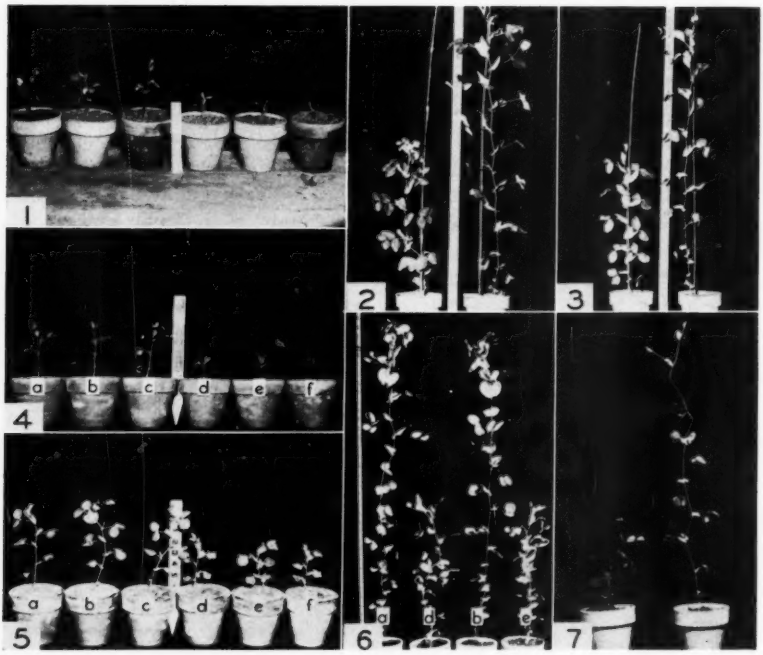
TABLE 2

SEEDLING HEIGHT AS AFFECTED BY EXPOSURE OF SEED TO INTENSITY LEVEL A FOR 20 SECONDS

Test	No. treated	No. germinated	No. of plants 10 cm. in height at 20 days	Control
1 (Sept. 27, 1951)	67	19 (29%)	3 (16%)	(20 or more seeds for each run; percentage germination 88%, all less than 10 cm. in ht. at 20 days.)
2 (Oct. 14, 1951)	79	17 (22%)	7 (41%)	
3 (Nov. 9, 1951)	75	27 (36%)	2 (07%)	
4 (Nov. 10, 1951)	88	21 (24%)	5 (24%)	
5 (Dec. 10, 1951)	71	26 (37%)	7 (27%)	
Total	380	110 (29%)	24 (22%)	

Five separate trials (Table 2), in which a total of 380 seeds were treated, resulted in an average of 29 per cent germination of which 22 per cent of the developing plants (not the seedlings used to determine root length) were over 10 cm. in height at twenty days, whereas none of the more than 100 control plants had attained this size (Figure 1).

Comparisons made between normal or control seedlings and elongated seedlings as to relative fresh weights demonstrated graphically that this difference in size was correlated with an increase in weight which was manifest early in the development of the plants (Figure 8). Twenty days after treatment the normal or control seedlings had attained a mean weight of 2.1 gm. whereas the mean weight



FIGURES 1-7
EXPLANATIONS IN TEXT

of elongated seedlings was 3.2 gm.; a difference of 52 per cent. This difference in fresh and dry weights increased with the age of the plants (Figure 9). Time to flowering, and average fruit weight, regardless of plant size, was essentially the same as that of the control plants.

At the end of thirty days the treated plants fell into three obvious categories—based on height and weight (Table 3). These groups were arbitrarily designated as a, b, and c. Group a, the largest number of plants, 79, were all less than 20 cm. tall and their average weight was 7.1 gm. Into group b fell those few plants, 7, which were between 21 cm. and 29 cm. tall. The mean weight of these seven plants was 9.9 gm. Group c included all plants 30 cm. or more

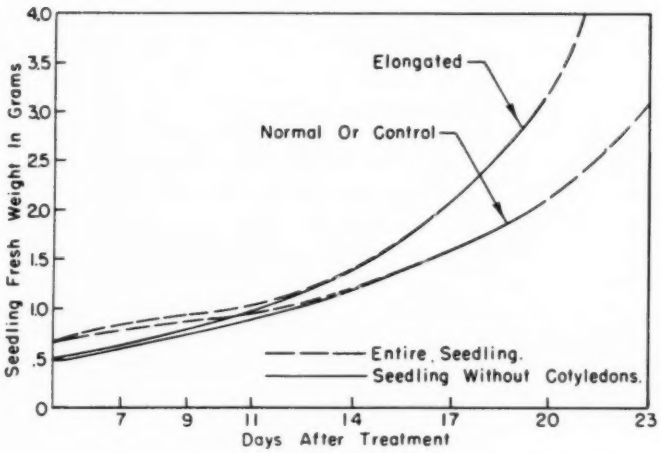


FIGURE 8

COMPARATIVE INCREASES IN FRESH WEIGHTS OF ELONGATED (TREATED) AND NORMAL OR CONTROL PEA SEEDLINGS

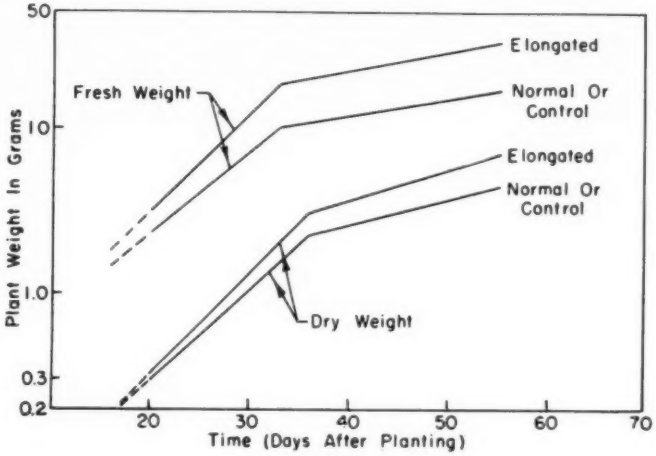


FIGURE 9

MEAN FRESH AND DRY WEIGHTS OF ELONGATED AND NORMAL OR CONTROL PEA PLANTS AT DIFFERENT AGES

tall. The 24 plants in this category averaged 12.3 gm. in weight. None of the control plants were over 20 cm. tall and a random selection of 40 plants averaged 7.3 gm. in weight. Three, or 12.5 per cent, of the plants of group c appeared etiolated in that their internodes were longer, their leaves were smaller and their average weights were less than other plants in this group (Figure 7; normal left, "etiolated", right. These etiolated plants flowered about eight days after the others and they set no viable seed.

When the plants of groups a, b, and c, and the controls were measured for height, when their second flowers appeared, the individuals of an averaged 34.5 cm. which was the figure obtained for the control plants (Figure 2, left). Group b averaged 52.3 cm. in height whereas the plants of group c, averaged 76.9 cm. in height (Figure 2, right).

TABLE 3

RELATIVE HEIGHTS AND AVERAGE FRESH WEIGHTS OF PLANTS AT 30 DAYS

Run	Group a 20 cm. or less	Group b 21 cm. to 30 cm.	Group c 30 cm. or more	Control
1	14 6.8 gm.	2 8.9 gm.	3 10.8 gm.	(All plants 20 cm.
2	9 6.5 gm.	1 9.3 gm.	7 11.0 gm.	or less, average
3	25 7.9 gm.	0 —	2 14.8 gm.	fresh weight, 7.3
4	12 7.2 gm.	4 10.5 gm.	5 12.5 gm.	gm.)
5	19 7.1 gm.	0 —	7 12.0 gm.	
	79 7.1 gm.	7 9.9 gm.	24 12.3 gm.	

B. *Effects of progeny from self-pollinated treated plants* (T_1).

Self pollination of the plants of groups a, b and c resulted in offspring comparable to their parents in development, height, and weight, with the exception of the progeny of group b. The latter plants gave rise to offspring indistinguishable from those arising from the selfing of either group a or the controls (Figure 3; control, left; elongated plant, right).

Normal appearing, treated plants set 619 seed which were 74 per cent viable. In thirty days these plants averaged 6.7 gm. in fresh weight and 14.2 cm. in height. At the time of flowering (second flower) their mean weight and mean height were 14.2 gm. and 33.6 cm., respectively (Table 4). The intermediate forms (group b) set 64 seed with a viability of 68 per cent. These self-bred plants differed little from the offspring of group a as to weight and height.

TABLE 4

T_1—Results of Selfing Plants From Exposed Seeds

			A random selection of 30 plants			
			At 30 days		At flowering	
T	No. of seed set	% viable	Ave. fresht. w	Ave. ht.	Ave. fresh wt.	Ave. ht.
Group a	619	74	6.7 gm.	18.1 cm.	14.2 gm.	33.6 cm.
Group b	64	68	6.5 gm.	17.7 cm.	13.1 gm.	30.9 cm.
Group c	291	78	12.0 gm.	37.4 cm.	28.0 gm.	77.8 cm.
Control	(200)	79	6.9 gm.	18.0 cm.	13.5 gm.	32.1 cm.

No significant difference among Group a, Group b, and control plants, since $X^2 = .025$; $P = 98\%$.

Elongated, treated plants set 291 seed having a germination percentage of 78 per cent. At thirty days these tall plants had an average weight of 12.0 gm. and their mean height was 37.4 cm. Upon flowering these tall plants gave an average of 28.0 gm. for weight and 77.8 cm. for height. The elongated offspring, like their treated parents, weighed almost double that of normal appearing or control plants. Also, these tall plants were well over twice the height recorded for the progeny of either group a or the control plants.

A second generation of self-pollinated plants gave results which were substantially the same as noted for the first generation. Without exception the viable seed from elongated plants gave rise to elongated progeny; whereas, the normal appearing, but treated plants produced normal sized offspring similar in all respects to control plants. At time of flowering a random selection of twenty, second generation selfed plants, had a mean height of 76.9 cm. and a mean weight of 27.4 gm. These plants, two generations removed from exposure to ultrasonic vibrations were employed in making the necessary crosses for analyses of the nature of expression of this elongate character.

C. Effects on progeny of crosses made with T_1 plants (F_1).

One hundred and seven crosses which ultimately set seed, involving elongated plants, normal appearing plants, and control plants were made (Table 5). These crosses, the results, and their numbers were as follows: ♀ elongate x ♂ elongate, 15 crosses gave all elongate (63); elongate x normal, 13 crosses gave all elongate (39); elongate x control, 12 crosses gave all elongate (41); normal x elongate, 17 crosses gave all normal (64); normal x normal, 13 crosses gave all normal (34); normal x control, 13 crosses gave all normal (26); control x elongate, 14 crosses gave all normal (38); and control x normal 10 crosses gave all normal (31). From these results it was ascertained that wherever the female used in a cross was elongated the progeny that developed displayed the same character. In fact, no cross produced elongated offspring save those in which the maternal parent possessed that character.

Figures 4, 5, and 6 are photographs of these crosses at 20 days after planting, 30 days after planting, and at maturity, respectively. In each figure the lettered plants denote the following crosses: a, elongate x elongate; b, elongate x normal; c, elongate x control; d, normal x elongate; e, normal x normal; and f, normal x control.

TABLE 5
CHARACTER EXPRESSIONS OF OFFSPRING FROM CROSSES BETWEEN ELONGATED AND NORMAL SIZE PLANTS OF T_1

	Male ♂		
Female (♀)	Elongate	Normal	Control
Elongate	Elongate (EE)	Elongate (EN)	Elongate (EC)
Normal	Normal (NE)	Normal (NN)	Normal (NC)
Control	Control (CE)	Control (CN)	——

TABLE 6
AVERAGE HEIGHTS AND WEIGHTS OF F_1 PLANTS (A RANDOM 10 PLANTS IN EACH CATEGORY)

	Height		Fresh weight		Dry weight	
Specimen*	20 days	50 days	20 days	50 days	20 days	50 days
EE	14.2 cm.	79 cm.	3.1 gm.	28.1 gm.	.30 gm.	5.7 gm.
EN	13.8 cm.	76 cm.	3.3 gm.	28.7 gm.	.34 gm.	6.1 gm.
EC	12.8 cm.	74 cm.	3.0 gm.	26.6 gm.	.33 gm.	5.6 gm.
NE	7.9 cm.	36 cm.	1.5 gm.	15.1 gm.	.24 gm.	3.9 gm.
NN	8.3 cm.	38 cm.	1.9 gm.	16.3 gm.	.28 gm.	3.7 gm.
NC	7.4 cm.	40 cm.	2.1 gm.	18.1 gm.	.28 gm.	3.6 gm.
CE	7.6 cm.	34 cm.	1.4 gm.	15.9 gm.	.23 gm.	3.2 gm.
CN	8.0 cm.	37 cm.	1.7 gm.	14.8 gm.	.27 gm.	3.8 gm.
Control	7.0 cm.	39 cm.	1.7 gm.	15.1 gm.	.24 gm.	3.9 gm.

*Refer to Table 5.

When measured 20 days after planting the progeny of the crosses that employed elongated females and pollen from elongate, normal, and control plants were all between 12.8 cm. and 14.2 cm. in height, while their fresh weights averaged between 3.0 gm. and 3.3 gm. and their mean dry weights were from .30 gm. to .34 gm. (Table 5, Figure 9). At the same time, none of the offspring from crosses made where the females were not elongate were over 8.3 cm. tall, and their fresh weight did not exceed 2.1 gm. nor their dry weight .28 gm. The surviving elongated products of these crosses at the end of fifty days were approximately twice the height and weight of either the normal appearing offspring or the control plants.

All of the progeny of the crosses were self-pollinated for two more generations and since the resulting plants did not differ from their parents no segregation was noted.

The log of plant fresh weight was plotted against the log of plant height and demonstrated that the development of elongated plants

was accelerated in comparison to normal plants or control plants in each of the three generations measured, T, T_1, and F_1 (Figure 10). Increase in size, height and weight, of the elongated peas of those three generations was essentially similar, but markedly distinct from that of the normal appearing plants. The development of normal treated plants and of control plants corresponded very closely. The accelerated development of elongated plants was expressed and easily detected at an early age (Figures 1, 4, and 8).

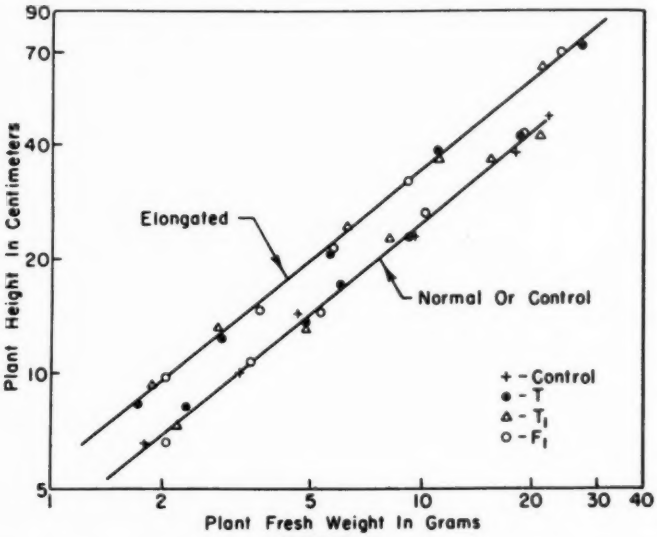


FIGURE 10

COMPARISONS BETWEEN ELONGATED AND NORMAL OR CONTROL PEA PLANTS BASED ON PLANT HEIGHT PLOTTED AGAINST PLANT FRESH WEIGHT

Comparisons made between elongated and untreated plants as to relative lengths and widths of internodes and as to cell size and cell number of these regions showed that the differences in height of the plants were correlated with an increased length of internode which was the result of increased cell length. In fact, the ratios between elongated plant height and its internodal cell length, and between normal plant height and its internodal cell length invariably came out to be as 1 : 1. Measurements were made on the epidermal

cells of plants 10, 30 and 60 days old. Transverse sections taken from the same regions of tall and normal plant stems revealed that the diameters of the taller stems were slightly less than those of the normal plants. Also, in transverse sections the areas displayed by the individual cells of the epidermis of elongated plants was somewhat smaller than shown by normal plants. The Avena test demonstrated a relative increase in activity for the tall plants of approximately 23 per cent over the normal.

D. Effects on some physico-chemical constituents.

Total nitrogen values (Table 7) on 20-day-old plants revealed that the elongated peas averaged 3.66 per cent nitrogen on a dry weight basis whereas the control plants averaged 5.33 per cent. At 40 days of age the elongated peas showed 2.58 per cent nitrogen and the controls 3.24 per cent. A few intermediate forms (Group b) were apparent at this time and they yielded a nitrogen percentage of 2.66.

TABLE 7
TOTAL NITROGEN AND PHOSPHORUS VALUES

Age Days	Specimen	% N per trial	Ave. % N	% P per trial	Ave. % P
20	Elongated	3.58 3.74	3.66	0.52 0.56	0.54
	Control	5.33 5.33	5.33	1.00 0.96	0.98
40	Elongated	2.58 2.58	2.58	0.27 0.27	0.27
	Intermediate	2.66 2.66	2.66	0.32 0.32	0.32
	Control	3.24 3.24	3.24	0.44 0.43	0.44
60	Elongated	2.34 2.46 2.28 2.32	2.35	0.47 0.46 0.48 0.46	0.47
	Intermediate	2.45 2.40 2.56 2.40	2.45	0.52 0.51 0.52 0.51	0.52
	Control	2.96 2.85 3.04 3.06	2.98	0.60 0.60 0.59 0.60	0.60

A further decrease in percentage of total nitrogen was evident in 60-day-old plants: the elongated peas averaged 2.35 per cent nitrogen, the intermediate group 2.45 per cent and the control peas 2.98 per cent.

At 20 days, 40 days, and 60 days the elongated peas yielded decreases in per cent of total nitrogen of 31 per cent, 20 per cent and 21 per cent, respectively, as compared to their controls. The intermediate plants, relative to controls, displayed decreases of 15 per cent and 18 per cent at 40 days and 60 days, respectively. The elongated plants showed a decrease of 36 per cent with an increase of age from 20 days to 60 days whereas the control plants demonstrated a relative decrease of 26 per cent.

Total phosphorus analyses of plants of the same ages yielded, on a dry weight basis, the following means: 20-day-old elongated plants and control plants, 0.54 per cent, and 0.98 per cent, respectively; 40-day-old elongated, intermediate, and control plants, 0.27 per cent, 0.32 per cent and 0.44 per cent, respectively; 60-day-old plants of the above groups, 0.47 per cent, 0.52 per cent and 0.60 per cent, respectively (Table 8).

The discrepancy in total phosphorus between elongated and control plants was most marked during the early development of the peas. Twenty-day-old, elongated plants yielded almost twice the percentage of phosphorus as noted for their controls. A difference of 39 per cent was found when the plants were 40 days old, but at 60 days the difference in phosphorus had dropped to about 22 per cent.

Treated plants that failed to develop the elongate character yielded approximately the same percentages of nitrogen and phosphorus as the control plants. Also, analyses for these elements on plants of succeeding generations, T_1 and F_1, gave yields comparable to those for the treated generations (T).

Free and hydrolyzed amino acid determinations on elongated plants failed to yield chromatogram patterns distinct from the "spots" given by control plants through the latter were more intense. Although distinct qualitative differences did not appear the variation in the intensity of the chromatograms suggests that a quantitative approach might well reveal differences of consequence.

Crude fiber of treated, elongated plants (T) based on percentage dry weight, increased from 7.80 per cent at 20 days to 12.51 per cent at 60 days while the control plants showed an increase from

TABLE 8

CRUDE FIBER PERCENTAGES (PERCENTAGE DRY WEIGHT)

	T						F_1	
	20 days		40 days		60 days		60 days	
Specimen	%/trial	Ave. %	%/trial	Ave. %	%/trial	Ave. %	%/trial	Ave. %
Elongated	7.79 7.81	7.80	11.69 11.81	11.75	12.79 12.28	12.51	12.31 12.18	12.22
Intermediate	9.07 8.99	9.03	15.46 15.47	15.47	16.61 16.15	16.38		
Control	10.23 10.17	10.20	16.01 16.09	16.05	17.10 16.98	17.04	16.83 17.04 16.75 16.81	16.85

10.20 per cent to 17.04 per cent in the same period (Table 8). In other words, during this 40-day period the elongated plants increased 60 per cent in crude fiber and the controls increased 62 per cent. The percentage of crude fiber in 60-day-old, elongated offspring of the crosses (F_1) was 12.22 and approximately the figure yielded by plants of the same age from the treated generation.

The protease of pea seeds increased slightly in activity up to 18 hours after soaking had commenced (Table 9). Seeds exposed to ultra sonic energy at this point commenced to give entirely different data for proteolytic activity than the seeds left untreated (Figure 11).

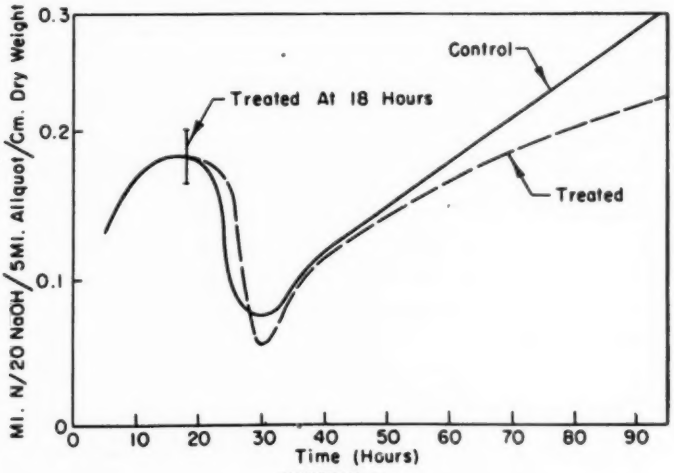


FIGURE 11

Protease activity of ultrasonized pea seedlings and their untreated controls. Activity is recorded as increase in ml. N/20 NaOH per gram fresh weight per ml. aliquot of digestion mixture after 24 hours' digestion at 40° C.

Six hours after treatment, or 24 hours after soaking, the exposed seeds yielded a mean figure of 0.06 (ml. n/20 NaOh per 5 ml. aliquot per gm. fresh weight), yet in the same length of time the activity of the control seeds had decreased to a mean of 0.04. The treated seeds demonstrated a rather sharp decrease in activity from 6 hours to 12 hours. In fact the determinations performed 12 hours following treatment showed these seeds had less proteolytic activity than the untreated ones. Minimum activity for either group of seeds

TABLE 9

TOTAL PROTEOLYTIC ACTIVITY AS EXPRESSED BY FORMAL TITRATIONS IN MILLILITERS

Number of hours after soaking seed	Initial			Final			Δ uncorrected	Δ corrected for weight
	Run 1	Run 2	Ave.	Run 1	Run 2	Ave.		
6	5.60	5.57	5.58	6.92	6.89	6.90	1.32	0.06
	5.73	5.70	5.71	7.13	7.13	7.13	1.37	0.06
12	5.78	5.76	5.77	7.35	7.30	7.32	1.55	0.06
	5.64	5.64	5.64	7.16	7.12	7.14	1.50	0.06
18	6.48	6.55	6.51	8.20	8.12	8.16	1.65	0.07
	6.52	6.50	6.51	8.20	8.16	8.18	1.67	0.07
	After 18 Hours One-Half of Remaining Seeds Were Treated							
Number of hours after treatment								
Treated	5.60	5.54	5.57	7.14	7.16	7.15	1.58	0.06
6	5.32	5.32	5.32	6.72	6.74	6.73	1.41	0.06
Control	5.32	5.30	5.31	6.36	6.12	6.13	1.09	0.04
	5.02	5.06	5.04	6.14	6.34	6.35	1.04	0.04

TABLE 9 (*continued*)

Number of hours after treatment		Initial			Final			Δ uncorrected	Δ corrected for weight
		Run 1	Run 2	Ave.	Run 1	Run 2	Ave.		
12	Treated	5.95	5.97	5.96	6.56	6.58	6.57	0.61	0.02
		5.80	5.82	5.81	6.24	6.20	6.22	0.41	0.01
	Control	5.91	5.91	5.91	6.48	6.47	6.47	0.56	0.02
		5.90	5.93	5.91	6.55	6.52	6.53	0.62	0.03
24	Treated	5.52	5.50	5.51	6.60	6.58	6.59	1.08	0.04
		5.50	5.50	5.50	6.58	6.54	6.56	1.06	0.03
	Control	5.46	5.50	5.48	6.54	6.50	6.52	1.04	0.04
		5.58	5.50	5.54	6.50	6.58	6.54	1.05	0.04
48	Treated	6.20	6.22	6.21	7.54	7.60	7.57	1.36	0.05
		6.08	6.06	6.07	7.60	7.50	7.55	1.48	0.05
	Control	6.02	6.06	6.04	7.60	7.64	7.62	1.57	0.06
		5.94	5.88	5.91	7.26	7.28	7.27	1.36	0.05
72	Treated	7.30	7.28	7.29	8.92	8.92	8.92	1.63	0.06
		7.14	7.10	7.12	8.86	8.80	8.83	1.71	0.06
	Control	7.22	7.28	7.25	9.36	9.30	9.33	2.08	0.07
		7.02	7.02	7.02	9.10	9.15	9.12	2.10	0.07

was recorded at this time. Further analyses pointed out increasing and diverging sets of data for the activity of treated seeds and of control seeds. This difference in pea protease was most marked 72 hours after exposure.

Discussion

Although reduced germination invariably accompanied exposure of soaked pea seed to intense ultrasonic vibrations, in some cases treatment seemingly induced an early acceleration of development, especially with those seeds given a high level dosage for 20 seconds. This developmental aberration of treated seeds was manifest as an increase in length of radicles which later was correlated with a decided increase in seedling height and weight as compared to control plants. Maturation of these elongated seedlings resulted in plants approximately twice normal size. Time to flowering and fruit yield was not affected and no vernalization resulted. Although Haskell and Selmen (8) noted reduced germination as the only genuine effect after field sowings following ultrasonic treatment of corn seed, Schwabe and Thornley (19) found a slight favorable effect on germination rate of winter rye seed. The later investigators reported no effect on time to flowering and found no vernalization. On the other hand, Istomina and Ostrovsky (10) claimed that pea seeds treated by ultrasonics matured earlier and had increased yields. In root tips of *Narcissus* and other plants a marked stimulation which produced longer roots than the controls was attributed by Newcomer and Wallace (16) to ultrasonic treatment of 400 Kc/sec.

These experiments are in accord with results reported by Ollivier (17) in that a dosage which promotes an increase in growth of seedlings is inhibitory or even lethal when applied for a longer duration; however, they are in contrast to those obtained by Loza (14) who concluded that root development is in no way favored by high frequency sound waves and that pea seeds are insensible to this type of radiation.

One of the more significant observations made during the course of these investigations was the earliness at which the difference in development between elongated seedlings and control seedlings was detected. A size difference was seen in seedlings less than a week old, and at 20 days the elongated seedlings were easily twice the size of

their controls. At this early stage even maximum varietal differences in development are not nearly as striking and varietal differences in growth are not as parallel nor do they usually give the same times to maturity.

The relative lengths of the internodes of tall and normal plants, where the former were found to be twice the size of the latter, were especially significant in view of the very close correlations that existed between these measurements and the lengths of internodal cells.

The maternal transmission and early expression of the induced elongate character would appear to be due to cytoplasmic effects. The evidence at hand suggests that the change is probably a dauermodification rather than an expression of either predetermination or a specific factor in the cytoplasm. Although dauermodifications are transmitted through a number of successive generations as expressions in the phenotype, there is a tendency for the character to become reduced in early generations. In these experiments an insufficient number of generations have been analyzed, as yet, to rule against a decrease in expression of the elongate factor. Caspari (4) considers dauermodifications to be environmentally induced. The direct effects of ultrasonic vibrations on living cells are of a purely physical nature—the induction of cavitation and the enormous acceleration of particles in the field. The production of high local heats, "point" heats, is a secondary effect attributed to one or both of the primary phenomena.

Energy of a sufficient magnitude to cause violent disruption of cellular contents should be ample to bring about intracellular chemical alterations. The immediate change in staining behavior of chromatin material (20) supports this contention. The acceleration of the particles and molecules may be as much as 500 km/sec and at high intensities liquids are ruptured and cavitation occurs. The effects produced by cavitation are manifold. A volume of water ruptured by cavitation leaves free valencies which probably accounts for the host of oxidation effects reported (22). Cavitation can bring about the emulsification of hitherto immiscible liquids. The coagulation of chromatin material may be due to the increased rate of movement of the individual particles which tend to accumulate wherever stationary waves occur, i.e. orthokinetic coagulation.

High local temperatures may be of significance in relation to the results of these investigations. Porter and his associates (18) re-

ported conversion of an acid azide into an isocyanate by sound energy. This is a case of readjustment of atoms within a molecule and it is believed that this rearrangement is accomplished by heat generated by gas bubbles.

Energy to rupture chemical bonds can be derived from the very great frictional forces set up by the relative motions of solvent and macromolecules in an intense ultrasonic field; however, Alexander (1) is of the opinion that denaturation of proteins in aqueous solution by ultrasound is probably not caused by the actual rupture of the molecules but by an unfolding of the globular shape under the tremendous shearing forces. When Laland, Overend and Stacey (13) attempted to isolate deoxyribonucleic acid (DNA) macromolecules from microorganisms by ultrasonic vibrations the irradiation broke some of the hydrogen bonds and disaggregated DNA to a less complex nucleotide which was highly susceptible to attack by the nucleotidase. However, Grabar and his co-workers (7) have shown that enzymes are often adversely affected by ultrasound. The effect of high frequency sound on pea protease could conceivably promote the detected decrease in proteolytic activity of the elongated plants.

In these investigations the determinations for total nitrogen, total phosphorus, crude fiber, and especially protease activity, together with the variation in these analyses, all lend credence to the view that the induced aberrations are chemical, cytoplasmic, and transmissible and apparently are expressed as a dauermodification of growth.

Summary

Intense untrasonic vibrations of 500 kilocycles per second and a power of 20 watts per cm.^{2} induced an apparent dauermodification in the growth of *Pisum sativum* L. The introduced morphogenetic variation, increased length of root and shoot axes, was first manifest as an accelerated development of radicles. This primary difference, which was soon accompanied by a marked increase in seedling height and weight, proved to be maternally transmissible. The elongated plants of the treated, self-bred, and F_1 generations were approximately twice the height and weight of the controlled plants. Close correlations among the lengths of shoots, internodes, and internodal cells were found and seemingly the differences in plant heights were due to relative cell sizes.

Significantly, this developmental variation was accompanied by heritable physico-chemical changes. Elongated plants yielded lower percentages for total nitrogen and total phosphorus than did untreated plants. Also, these taller plants gave a lower yield for crude fiber. Protease activity of treated pea seeds and the subsequent seedlings was unlike that of the controls. In fact, in the period 12 hours to 72 hours following treatment total proteolytic activity of exposed peas was markedly lower than control samples.

Although ultrasound caused an environmentally induced developmental aberration, time to flowering, and fruit and seed yields were not affected. In fact, the only gross morphological variation detected was that of increased size.

The evidence presented suggests that ultrasonic vibrations have provoked maternally transmitted changes in the biologic systems of exposed peas. These alterations are shown in the development and in the physico-chemical analyses of the plants and probably constitute a dauermodification of growth.

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THE EFFECT OF PIGEON CROP-MILK ON GROWTH IN CHICKENS

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INTRODUCTION

It has been known for many years that the young of certain birds have a very rapid growth rate. Riddle (1928) and Bates, Riddle, Lahr, and Schooley (1937) report that the squab appears to excel other vertebrates in rate of growth. At hatching time squabs weigh, on the average, about 18 grams and 27 days after hatching the weight may increase to 430 grams or more. Although several hypotheses are advanced to explain this phenomenal growth rate, the basic reasons are not yet understood.

John Hunter in 1786 (according to Tegetmeier, 1868) was the first to make note of the fact that pigeons and doves (males as well as females) feed their young by regurgitating a substance which may be called a curd and which is now referred to as "crop-milk". More recently, Levi (1941) notes that the African sand grouse, which is related to the pigeon, also produces a crop-milk and feeds its young by regurgitation. These are the only species, however, known to produce such a substance in their crops.

Litwer (1926) found that the "milk" is produced by desquamation of the epithelial cells lining the crop (not a secretion as in the production of mammalian milk). The crop wall of the parent birds becomes very thick until by hatching-time the lumen of the crop is nearly filled with projecting rugae. Beams and Meyer (1931) and Patel (1936) agree with Litwer in this respect. Riddle and Braucher (1932), Riddle, Charles and Cauthen (1932), Dabrowska (1932), Schooley, Riddle, and Bates (1937), Champy and Coujard-Champy (1945) and Mietes and Turner (1947) all agree that a hormone, prolactin, is instrumental in the proliferation of the crop epithelium.

For the first few days after hatching the young squab receives

a diet of almost pure crop-milk. Usually, it is not until the 3rd to 5th day after hatching that "hard foods" such as grain begin to appear in any quantity in the squab's crop. From this time on, there is a gradual decrease in the relative quantity of "milk" as compared to hard food in the crop of the squab.

The work of Carr and James (1931) and Guareschi (1936) indicates that the crop-milk of pigeons contains a factor or factors important to the rapid growth of their young. Nothing is known, however, concerning the identity of any such factors.

Because of the very effective action of the growth-promoting factor in the crop-milk of pigeons on their young, the question arose as to the possibility of its effect on the young of other birds. It was for this reason that the following investigations were carried out.

Material and Methods

Homer pigeons were used as a source of crop-milk for these investigations. They were placed in a loft that had been thoroughly cleaned and provided with at least twice as many nesting boxes as there were pairs of pigeons. This was done to discourage fighting. Long pine needles were scattered on the floor of the loft for nesting material. They were fed a commercial pigeon chow, entirely graniverous in composition.

After the squabs had hatched, and after they were fed by the parent birds several methods for obtaining the crop-milk were attempted. At the time, the most appropriate method was that in which an incision was made directly through the crop wall of the squab immediately after the feeding. The "milk" or "curd" was gently forced through the opening into an Erlenmeyer flask, and then refrigerated at 1°C. During the first day after hatching the crop-milk is quite fluid, but from that time on becomes much less fluid and somewhat resembles cottage cheese. The incision was closed by means of a braided silk suture, size 2-0. The squab was then placed back in the nest to be used again later the same day. Usually "milk" was collected from each squab twice a day for a period of 3 to 5 days at which time it was found that the grain content of the crop was too great for efficient separation from the milk. Then the squabs were destroyed so that the parent birds would mate again.

The best time for collecting the milk was ascertained by observing the feeding habits beginning early in the morning. However, there

does not appear to be any "regular" feeding time. Early morning feedings were made between 6 and 8 A.M. and afternoon feeding between 2 and 5:30 P.M. This was true except when the milk was removed from the crop of the squab, which, on replacing it in the nest, almost immediately was fed again by the parent birds.

Results

After a sufficient quantity of milk was collected, tests were made on young White-Rock chicks. When they were 1 day old they were weighed and separated at random into two groups, each group consisting of 13 chicks. No attempt was made to separate the groups by weight or sex. Two chicks died in each group before the experiment was terminated. This left 11 chicks in Group A, 6 males and 5 females and 11 chicks in Group B, 5 males and 6 females.

Group A was the control group which received only the base ration. This ration has been in use for some time in the Department of Poultry Husbandry at the University of Nebraska and is given in Table 1.

TABLE 1

Base Ration Fed to the Control Chicks (Group A)

Yellow corn meal	56 lbs.
Corn gluten meal	10 lbs.
Soy bean meal	25 lbs.
Alfalfa meal	5 lbs.
Act. animal sterol (premix 200)	1 lb.
Mineral mix No. 50	3 lbs.

The experimental group (Group B) received the same ration plus raw pigeon milk which was mixed with the base ration so that each chick received on the average about 5 grams of "milk" per day. The supply of crop-milk was exhausted after 6 days and the chicks from then on were placed on the same ration as those of Group A.

The chicks were weighed daily for the first 4 weeks although only weekly weights are given in the table. They were not weighed, however, between the 4th and 6th weeks. The ration allowed each group was also weighed and recorded.

When the chicks were 1 day old, those of Group A had an average weight of 37.4 grams while those of Group B averaged 38.7 grams. (See Table 2.)

During the first 3 days there were no great differences in weight gain but from the 4th day on it is quite evident that the greatest gains always occurred in the chicks which were fed the base ration plus crop-milk (Group B). These chicks also showed a more rapid over-all development; their feathers appeared earlier than those of the chicks in Group A; their combs showed greater development; and greater body size was obvious when compared with the controls.

From the results presented in Table 2, it is evident that there is a progressive increase in growth in the experimental as compared to

TABLE 2

THE WEIGHTS OF WHITE ROCK CHICKS OVER A 6-WEEK PERIOD WHEN FED "A", THE BASE RATION AS GIVEN IN TABLES 1 AND "B" BASE RATION PLUS PIGEON CROP-MILK FOR THE FIRST 6 DAYS

(Weight is given in grams)

	Age in days					
Sex	1	7	14	21	28	42
	Group A—Fed Base Ration Only					
Female	33.5	53	90	137	200	265
Female	34.0	57	84	121	161	261
Male	35.0	61	91	141	190	315
Male	35.0	60	105	172	256	381
Male	36.5	56	100	182	274	447
Male	37.0	55	86	136	189	245
Female	37.5	60	100	146	210	319
Female	37.5	60	106	156	206	330
Male	38.0	52	73	120	178	227
Male	40.0	72	123	182	242	322
Female	45.5	75	114	179	256	342
Average weight	37.4	59.8	95.9	148.0	214.7	314.0
Av. Wt. Gain/wk.	——	22.4	36.1	52.1	56.7	*99.3
Sex	Group B—Fed Base Ration Plus Pigeon Crop Milk for First 6 Days					
Female	34.0	83	148	248	348	534
Female	35.5	62	114	174	256	376
Female	35.5	64	128	204	289	443
Male	38.0	60	108	148	198	343
Female	38.0	69	136	212	301	374
Male	39.0	82	152	216	274	387
Male	39.0	73	140	211	304	501
Female	41.0	76	148	234	318	484
Female	41.0	74	144	222	301	447
Male	41.5	72	142	212	306	452
Male	41.5	78	138	212	300	437
Average weight	38.7	71.6	130.8	197.8	290.4	434.3
Ave. Wt. Gain/wk.	——	32.9	58.7	67.5	92.6	*143.9

*Weight gain for 2 weeks.

the control chicks for the six-week period although the crop-milk was fed only during the first 6 days.

The total average weight of the control chicks, fed only on the base ration over a six weeks' period was 314 grams while the average weight of those fed base ration and crop-milk was 434.3 grams. Thus, the experimental chicks weighed about 38% more than the controls at six weeks of age.

If chicks of Group A and chicks of Group B with more or less comparable starting weights are compared daily it is found that in all but two cases the chicks of the B group gained more weight than those of Group A.

It was noted that the chicks which were fed the crop-milk ate more of the ration fed them, than the control chicks did. This could be expected since assimilative processes, as are required during such rapid growth, would naturally necessitate a greater food consumption. The question then arises as to why the test group eats more than the controls.

Discussion

The different methods of analyses that have been used for ascertaining the composition of pigeon crop-milk are rather inconclusive. According to Dabrowska (1932) and Davies (1939) dried crop-milk consists approximately of 58% protein, 34% fats, and 8% minerals, etc. There are no carbohydrates whatever in the milk. The mineral content is characterized by a high potassium and phosphorus level and low sodium and chlorine. Reed, Mendel and Vickory (1932) find that there is a rather low Vitamin A and B complex activity shown by crop-milk. Despite the low concentration, they suggest that the rapid growth of squab may be due to the presence of these vitamins. Dabrowska (1932) suggests that this rapid growth rate may be due to the high protein and fat content of the crop-milk. Guareschi (1936) however, fed squabs on an artificial diet which was high in protein, fat, and Vitamin B and found that they did very poorly on it.

The suggestion made by Dabrowska certainly does not hold for the young chicks that were used in the present investigation. They were fed the usual base ration plus 5 grams of pigeon crop-milk for the first 6 days after hatching. The amount of protein and fat added would therefore be insufficient to result in such a growth rate

which continued for many days after the feeding of crop-milk was discontinued.

It is possible that the growth-promoting action may be due to a vitamin since many vitamins may be stored in the body and exert their actions over long periods of time.

It would be interesting to ascertain the effect of feeding crop-milk throughout the growing period of the chicks. This, however, is difficult because of the large quantities of "milk" needed. At any rate, it is evident that crop-milk does contain something that stimulates growth in chicks but more extensive investigations are needed to ascertain its nature, concentration and "modus operandi".

Conclusions

1. A method for obtaining pigeon crop-milk from the squab immediately after feeding is described.
2. After sufficient quantities were obtained (by storing at 1°C) the crop-milk was fed to 11 day-old White Rock chicks weighing an average of 38.7 grams as compared to 11 control chicks averaging 37.4 grams.
3. Crop-milk equivalent to 5 grams per chick per day for 6 days was added to a basic ration and the daily growth of these chicks was compared with that of controls fed only the basic ration.
4. The chicks fed the "milk" showed a more rapid overall development, their feathers appeared earlier, their combs were larger, and, from the third day on, growth was more rapid than in the controls.
5. At the end of six weeks, the total average weight of the chicks fed crop-milk was 434 grams as compared to 314 grams for the controls.
6. It is suggested that a growth-promoting factor or vitamin is present in pigeon crop-milk which effects the growth of young chicks as well as squab.

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SINGLE SYSTEM FOR MEASURING GROWTH RESPONSES OF THREE ORGANISMS TO FOLACIN AND RELATED FACTORS (BACTERIMETRIC STUDIES. VII.)*

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Folacin (pteroylglutamic acid) and related factors are commonly measured by the growth response of either Streptococcus lactis R 8043 or Lactobacillus casei 7469; or, for differential characterization, both of these organisms. The well established methods (6, 5) of Teply and Elvehjem (1945) and of Roberts and Snell (1946) use for S. Lactis R a medium based on acid-hydrolyzed casein, incubation at 30° and determination of growth response by titration, while for L. casei a medium containing enzymatic casein hydrolysate, incubation at 37° and turbidimetric measurement of growth responses are employed. We briefly described (1949) a combination of growth medium, incubation and growth measurement which is suitable for both organisms and thus simplifies their simultaneous analytical use (11).

The present paper gives a full account of this method after it has been further improved and simplified and, conditions having been stabilized, two years of experience with it have been accumulated.

EXPERIMENTAL

Folacin Standards.—Folacin has been shown (1) to be much more stable in alkaline than in acid solutions. The following routine system of preparing standard solutions has consistently given reliable results.

Standard I. A 5.40×10^{-4} *M* solution of folacin[1] in 10^{-2} *M* NaOH, prepared fresh every three months.

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Standard II. A 5.40×10^{-6} *M* solution in 10^{-4} *M* NaOH, prepared every month by dilution of Standard I.

Standard III. A 5.40×10^{-8} *M* solution in 10^{-6} *M* NaOH, prepared every week by dilution of Standard II.

Standard IV. A 5.40×10^{-10} *M* solution in 10^{-8} *M* NaOH, prepared every two days by dilution of Standard III.

Standard V. A 1.80×10^{-10} *M* solution prepared from Standard IV by dilution with 2 volumes of water.

All solutions are kept cold. Because of the million fold concentration range used special safeguards against contamination are advisable. Flasks and pipettes are soaked in chromic-sulfuric fluid, thoroughly rinsed and finally autoclaved in water (9). Besides, containers are identified and re-used for the same concentration ranges. Standard I, whose preparation involves handling of dry folacin, is always prepared one day before, and never in the same room in which dilutions are made.

Medium.—The chief nitrogen source is a commercially available enzymatic casein hydrolysate: Casein Hydrolysate Enzymatic, 5 per cent, vitamin free, Nutritional Biochemicals Corporation, Cleveland 28. Supplementary amino acids, minerals, nucleic acid components and vitamins are used in the form of nine solutions which are kept in the refrigerator and renewed after six months. These are described in Table 1. Basal medium is made as follows: For each 100 cc. weigh out 4.1 gm. (50 mM) anhydrous sodium acetate, 4.0 gms. glucose, 696 mg. (4 mM) K_2HPO_4, 680 mg. (5 mM) KH_2PO_4, and 50 mg. ascorbic acid. Dissolve *without heat* in approximately 25 cc. H_2O and add 2 cc. of each of the nine solutions listed in Table 1, then 25 cc. of the specified casein hydrolysate. Add the amount of 1 *N* NaOH (from 15 to 25 cc.) required, according to previous titration (7), for a final pH of 6.45 and make to volume. Check the pH electrometrically. It should be between 6.40 and 6.50. In order to obtain a completely clear medium, it is left in the refrigerator overnight and then filtered through sintered glass (M filter). Medium is made when needed, but apparently retains full activity for at least three months when stored in the frozen state.

Culture Conditions and Readings.—These are as previously described (10, 11): calibrated tubes with 6 cc. finished medium containing 3 cc. basal medium are sterilized for 2.5 minutes at 120°; measurements are made in the Coleman spectrophotometer at 675 mμ, color blanks being determined before incubation; the tubes are

incubated in a water bath of 37.7±0.05°; net turbidity readings (color blanks subtracted) are converted to AOD (adjusted optical density) values, to correct for deviations from Beer's law, by means of a table.

Inocula.—The medium for inoculum growth consists, per tube, of 3 cc. basal medium and 3 cc. Standard IV. A supply of such tubes, plugged with cotton, sterilized and covered with Parafilm, may be stored for a month's use in the freezing compartment of the refrigerator. For each of the two organisms one tube is inoculated from a standard agar culture. The tubes are incubated, read periodically and plunged into ice water when AOD 120 has been reached. The next day 0.2 cc. of each culture is transferred to other inoculum tubes, which again are allowed to grow to AOD 120. Semilogarithmic plotting of readings taken periodically yields linear growth rates. After several successive cultures these become constant with division times of approximately 1.0-1.2 hours for L. casei and 0.8-0.9 hours for S. lactis. The inoculum for actual assay work consists of 1 drop per tube of such standardized log phase cultures grown on the day of the experiment (the cultures may be stored in an ice bath overnight with little or no loss of activity). The method of inoculum growth is illustrated by Figure 1. It combines the advantage of high activity (log phase cells) and low blank value (the amount of folacin carried over with the inoculum can be estimated).

Standard Responses (*Folacin*).—Each condition of measurement (standards or unknowns) is represented by two tubes and each tube is read twice, ordinarily with an interval of five hours. By inoculating the L. casei tubes one-half hour earlier than the S. lactis tubes and reading them one-half hour later the next day, one conveniently obtains for L. casei measurements after 18 and 23 hours of incubation and 17- and 22-hour measurements for S. lactis. To permit rational comparison of different compounds (folacin, teropterin, etc.) concentrations are expressed on a molar basis: 1 tM (one trillimol), i.e. 1×10^{-12} mol, equals 0.441 mγ folacin (11), etc. Folacin standard tubes for L. casei contain 0, 1, 2 and 3 cc. of Standard V per 6 cc. tube, and those for S. lactis contain the same quantities of Standard IV. Representative response curves obtained under these conditions are shown in Figures 2 and 3. The average difference between the readings of duplicate tubes, standards or unknown, is approximately 7 AOD units.

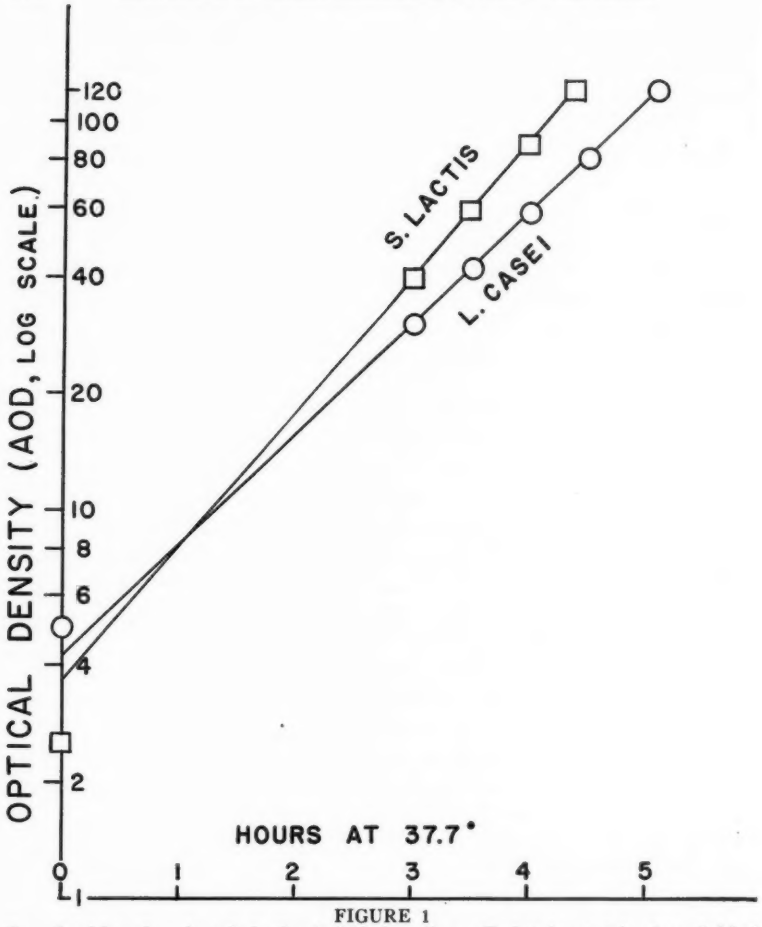

FIGURE 1

Growth of log phase inocula in the analytical medium. Each tube contains 6 cc. finished medium including 3 cc. basal medium and 0.71 mγ folacin (0.27 tM per cc.).

Responses to Teropterin.—The response of L. casei to teropterin[1] is always represented by a typically concave curve (cf. (3)) so that

[1]We are indebted to Dr. James M. Smith, Jr., of the Calco Chemical Division, American Cyanamid Co., Bound Brook, N. J., for analyzed samples of folic acid (pteroylglutamic acid 92%, p-aminobenzoylglutamic acid 0.5%, H_2O 7.5%) and teropterin (sodium pteroyltriglutamate 83%, sodium p-aminobenzoyltriglutamate 3%, H_2O 12%). These preparations have served as our primary standards, with effective molecular weights of 480 and 948 respectively.

the ratio between teropterin-induced and folacin-induced growth decreases with decreasing molar concentration. However, the absolute amount of the responses as well as their ratio vary from experiment to experiment. Examples of responses to teropterin are included in Figures 2 and 3.

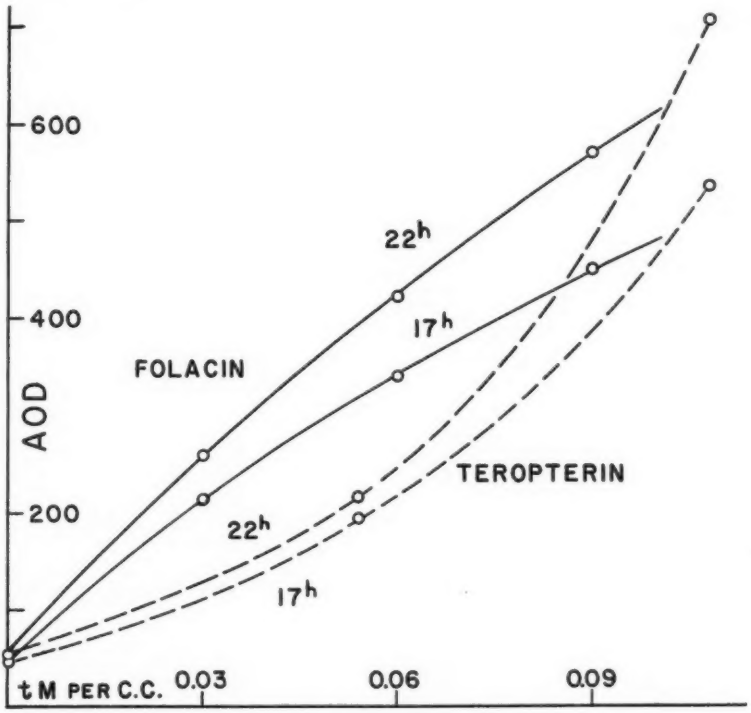

FIGURE 2
Standard responses of L. casei to folacin (full lines) and teropterin (dash lines).

Discussion

Our principal experience with the present analytical method has been in the study of folic acid-like factors in human blood, described elsewhere (8), in which L. casei and S. Lactis R were principally employed. However, Leuconostoc citrovorum 8081 also grows under the

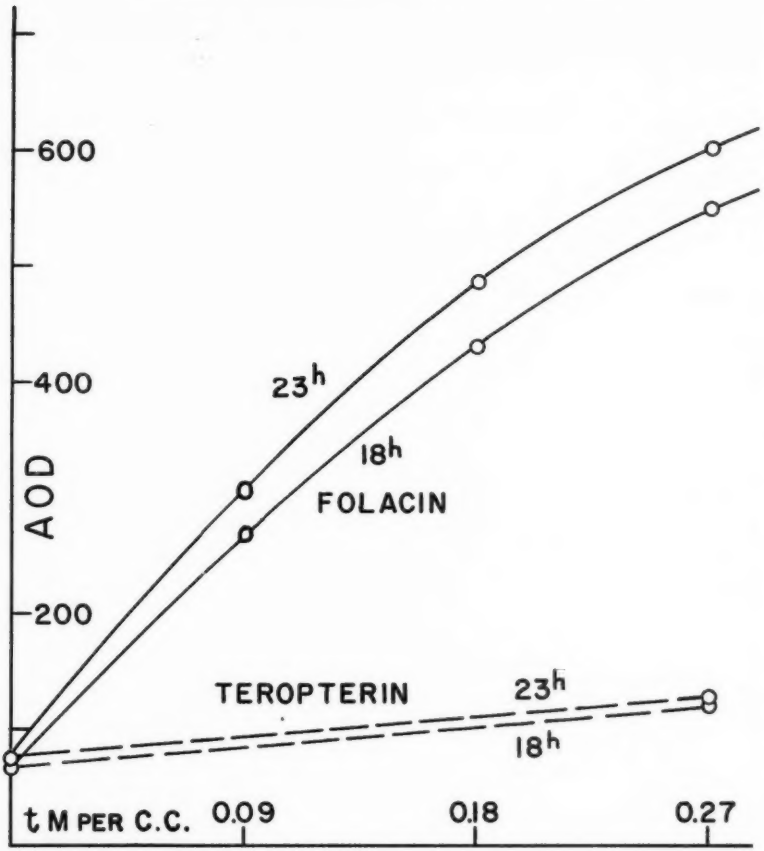

FIGURE 3
Standard responses of S. lactis R to folacin (full lines) and teropterin (dash lines).

conditions described, provided citrovorum factor is supplied;[2] the system thus is equally suitable for the determination of folinic acid or citrovorum factor.

[2]L. citrovorum 8081 from a standard agar culture grew in the present medium reinforced with 5 mγ of Leucovorin (Lederle) per cc. Several transfers in the liquid medium containing 0.5 mγ Leucovorin per cc. were necessary to obtain an inoculum of low blank response. Inocula grew with division times of 0.8-1.0 hours. Nearly linear responses were obtained, after 17 and 23 hours, over the range of AOD 40 to 700 with 0 to 0.425 mγ Leucovorin per cc.

The composition of our medium is based on the L. casei media of Teply and Elvehjem (6) and of Roberts and Snell (5). The lower casein content of our medium (0.625 per cent instead of 2.0 per cent in the medium of Roberts and Snell) is based on experiments which showed that response to folacin is most nearly independent of the concentration of casein hydrolysate when the casein content is between 0.5 and 0.75 per cent. Higher casein concentrations tended to produce somewhat slower growth and erratic responses.

The incorporation of ascorbic acid, suggested by the work of Rickes, Koch and Wood (4), is based on experiments with graded concentrations which showed that the vitamin enhances the growth rate of both organisms, although with higher concentrations the effect is reversed in the case of S. lactis. Supplementations with asparagin, glutamic acid and serine were not found beneficial.

Careful control of the initial pH and attention to the buffering capacities of medium and added analytical specimen are important for optimal results, as shown in another study (7).

The main advantage of the present system is the dispensability of separate technics for the different organisms, without sacrifice of sensitivity for either. Our growth responses appear to be equal or superior to those of published methods which have separate specifications for the two organisms. This is shown in Table 2 which summarizes a few characteristics of selected methods.

The folacin response curves shown in Figures 2 and 3 represent actual records which are close to the mean response of approximately 80 experiments with L. casei and approximately 30 with S. lactis. Responses are not identical from day to day but vary within the extremes of approximately 70 and 140 per cent of those plotted. Individual characteristics of different manufacturing lots of the casein hydrolysate are the main factors of variability, the responses of different media prepared from a single lot falling within a distribution range of approximately ± 20 per cent. Careful standardization of inoculum, pH and incubation time minimizes but does not eliminate variability.

TABLE 1
STOCK SOLUTIONS FOR FOLIC ACID MEDIUM

Label	Substance	Amount	Added Solvent	Final Volume
ALA	*DL*-Alanine	5.00 g		250 cc.
ASP	*L*-Asparagin	2.50 g		250 cc.
CYS	*L*-Cystine	5.00 g	100 cc. 2 *N* HCl	250 cc.
SAL	$MgSO_4.7\ H_2O$	5.00 g		
	NaCl	250 mg		
	$FeSO_4.7\ H_2O$	250 mg		
	$MnSO_4.4\ H_2O$	250 mg	1 cc. 2 *N* HCl	250 cc.
AGU	Adenine.H_2SO_4	435 mg		
	Guanine.HCl	310 mg		
	Uracil	250 mg	25 cc. 2 *N* HCl*	250 cc.
XAN	Xanthine	250 mg	10 cc. conc. NH_4OH	250 cc.
VIT	Nicotinic acid	50 mg		
	Ca pantothenate	50 mg		
	Thiamine HCl	50 mg		
	Pyridoxamine HCl	20 mg		
	p-Aminobenzoic acid	10 mg		1,000 cc.†
RIB	Riboflavin	12.5 mg	0.75 cc. acetic acid	250 cc.
BIO	Biotin	10 mg	2 cc ethanol, then 0.01 *N* HCl	10,000 cc.†

*Use heat to dissolve.
†Save 250 cc. and discard remainder or prepare 250 cc. by dilution of an aliquot of higher concentration.

TABLE 2
GROWTH RESPONSES TO FOLACIN IN DIFFERENT METHODS

Author and year	Ref.	Incubation Time hrs.	Incubation Temp. °C.	Measurement	Folacin conc. for approx. halfmax. response mγ/cc.
	(a)	Lactobacillus casei			
Teply & Elvehjem '45	(1)	30-72	30	titrimetric	0.04
Roberts & Snell '46	(2)	16	37	turbidimetric	0.04
Rickes *et al.* '49	(10)	17	37	turbidimetric	0.4
Flynn *et al.* '51	(11)	40	33	turbidimetric	0.015
Present '52		18	38	turbidimetric	0.02
	(b)	Streptococcus lactis R			
Teply & Elvehjem '45	(1)	30-72	30	titrimetric	0.4
B. C. Johnson '46	(8)	16	30	turbidimetric	0.15
Dick *et al.* '48	(4)	72	30	titrimetric	0.06
Flynn *et al.* '51	(11)	18	33	turbidimetric	0.15
Present '52		17	38	turbidimetric	0.06

Summary

For the determination of folacin and the analytical differentiation of related factors, *Lactobacillus casei* and *Streptococcus lactis R* have in the past been used under separate culture conditions. A single system is now described which has been found suitable for the rapid and precise measurement of compounds of the folic acid family with *L. casei* 7469 or *S. lactis* R 8043. It can also be used for the determination of folinic acid with *L. citrovorum* 8081. The medium requires four dry substances, ten stock solutions of chemicals, and a commercial enzymatic casein hydrolysate. Instead of inoculum cultures grown in special media, log phase inocula grown in the analytical medium are employed. All growth responses are measured turbidimetrically after 17 to 23 hours of incubation at 37.7°. The sensitivity of the method is equal to or better than that of other procedures using separate culture conditions: the folacin concentration of half-maximum response is for *L. casei* approximately 0.02 mγ per cc., and for *S. lactis* R approximately 0.06.

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12. Doe, J. 1942. The preparation of manuscripts. *Growth*, **6**, 700-725.

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12. Doe, J. 1942. The Preparation of Manuscripts. New York: Holt. Pp. 400.

In the text, references should be made to the author and either the year or reference number, e.g., Liebig and Wöhler (1837) or Liebig and Wöhler (12),—or still more exactly, Liebig and Wöhler (12, p. 15). Summary and conclusions should be included; footnotes should be avoided. Changes from copy will be charged to the author.

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*The Editors regret that, due to increased publication costs, it has been necessary to increase charges temporarily by 15 per cent.

TABLE OF CONTENTS

Paper No.		Page
422	Growth factors in alcoholic extracts of chick embryos By Morgan Harris	215
423	A theory of differentiation By Gairdner B. Moment	231
424	Effects of intense ultrasonic vibrations on *Pisum*. I. Effects on root meristems By John L. Spencer	243
425	Effects of intense ultrasonic vibrations on *Pisum*. II. Effects on growth and their inheritance By John L. Spencer	255
426	The effect of pigeon crop-milk on growth in chickens By Donald M. Pace, Paul A. Landolt, and Frank E. Mussehl	279
427	Single system for measuring growth responses of three organisms to Folacin and related factors (Bacterimetric studies. VII) By Gerrit Toennies, Henry G. Frank and Dorothy L. Gallant	287